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RuvBl2 as a regulator of hTERT

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Submitted in February 2010 to the The Royal College of Surgeons in Ireland for the degree of Doctor of Philosophy

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Table of contents

Candidate Thesis Declaration

I declare that this thesis, *which I submit* to RCSI for examination in consideration of the award of a higher degree PhD is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

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Acknowledgements

I would like to express my deep gratitude to Dr Leonie Young for her supervision. I am grateful to Dr Aisling Redmond, Dr Marie McIlroy, Ms Fiona Bane, Ms Sinead Cocchiglia and all other members of the molecular endocrinology laboratory for their invaluable assistance provided during the last 2 years. I am grateful to Professor Roger Reddel for the 6G and 1-3C cells (and their initial characterisation as described in chapter 3) and to Dr Achim Treumann for the mass spectrometry. I would also like to thank Dr Kilian Perrem for supervising me during the first 2 years of the project.

Abstract

Telomere maintenance is essential for the continued proliferation of tumour cells and is performed through the mechanism of telomerase activation or by a recombination-based process referred to as alternative lengthening of telomeres (ALT). The most important components of active telomerase are the reverse transcriptase enzyme (hTERT) and the non-coding RNA subunit (hTR). hTERT is the rate-limiting component and is highly regulated. The detection of genes that regulate either telomere maintenance mechanism is therefore of potential therapeutic significance. To address this, the study used stable isotope labelling of/with amino acids in cell culture (SILAC) to compare the proteomes of 2 cell lines, 6G and 1-3C that had been previously derived from a single SV40-immortalised jejunal fibroblast cell. Both cell lines are therefore genetically similar except for the fact that the 6G and 1-3C lines use different TMMs (telomerase and ALT respectively) to counteract telomere attrition. An ATPase-dependent DNA helicase protein called RuvBl2 was found to be relatively overexpressed in the 6G cell line. RuvBl2 is known to be involved in the control of gene expression and a putative role in hTERT regulation was investigated. siRNA knockdown of RuvBl2 reduced hTERT protein levels in the 6G cell line. RuvBl2 was able to interact with the hTERT promoter and was also shown to upregulate hTERT gene transcription. Collectively the data in this study suggest that RuvBl2 is a positive regulator of hTERT gene expression and this observation supports the existing body of data that RuvBl2 has a multitiude of functions that contribute to the tumorigenesis.

Posters of research presented

IMSS (Irish mass spectrometry society) annual general meeting 2007

'The identification of novel regulators of telomere maintenance mechanisms in immortalised human fibroblasts by SILAC-based proteomic analysis.'

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IACR (Irish Association of Cancer Research) conference 2009

'Telomerase and cancer: The identification of novel candidate proteins involved in the regulation of telomerase activity in immortalised human fibroblasts by SILAC-based proteomic analysis'

Padraic Flavin[1,2], Aisling Redmond[2], Marie McIlroy[2], Fiona Bane[2], Sinead Cocchiglia[2] Achim Treumann[3], Christian Köhler[3], Roger Reddel[4], Elaine Kay[1], Mary Leader[1], Kilian Perrem[1], Leonie Young[2]

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List of figures

List of tables

Chapter 1

Introduction

1.1. The telomere

Telomeres are nucleoprotein structures situated at the ends of chromosomes. The structure of a telomere can be divided into its nucleic acid and protein components. Each of these elements will be discussed separately.

1.1.1. Nucleic acid

The nucleic acid component of human telomeres consists of an array of TTAGGG hexanucleotide microsatellite repeats and a recently discovered associated RNA species called telomere repeat RNA (TERRA). The telomere-associated DNA sequence can be defined as the contiguous stretch of $(TTAGGG)_n$ repeats at the chromosomal terminus (Moyzis et al., 1988; Meyne et al., 1989). The typical length of this sequence varies from 5-15 kilobases (kb) in somatic cells (Figure 1.1). Telomere lengths usually range from a maximum of 20kb in actively dividing cells that constitute germline tissue to a minimum of 2kb in cells that have exhausted their mitotic potential (Levy et al., 1992). Telomere length also depends on the particular chromosome. For example, single telomere length analysis (STELA) was used to measure telomere lengths of specific chromosomes in a panel of senescent fibroblasts and it was found that the 17p telomere has a modal length of only 300bp (Britt-Compton et al., 2006). A modified version of the STELA assay was used to analyze Xp and Yp telomere lengths in cancer cells and this led to the discovery of 't-stumps' that consist of at least seven hexanucleotide repeats (Xu and Blackburn, 2007). This is the shortest sequence currently known that is sufficient for telomeric complex formation.

The terminal part of the telomere is the 3' G-strand ssDNA overhang that is ~100-200 bases long (McEachern et al., 2000; Makarov et al., 1997). This ssDNA terminus folds back on its self and invades the duplex telomeric DNA to form the T-loop structure (Wellinger and Sen, 1997; Griffith et al., 1999; Nikitina and Woodcock, 2004). This structure prevents the exposure of the end of the telomere and makes it less susceptible to DNA repair or nuclease-mediated degradation events. Adjacent to the terminal section of the telomere solely composed of these microsatellite repeats is a region which has a variable composition of these hexanucleotide sequences interspersed with very similar sequences that usually differ by a single nucleotide e.g. (TGAGGG)n (Baird et al., 1995; 2000). The DNA sequence flanking this is comprised mainly of sequences which are homologous to the telomere-associated repeat-1 (TAR1) family of DNA repeat sequences (Brown et al., 1990). The remainder of the subtelomeric sequence consists of segmental duplications and these represent ~80% of the most terminal 100kb of a human chromosome (Riethman et al., 2003).

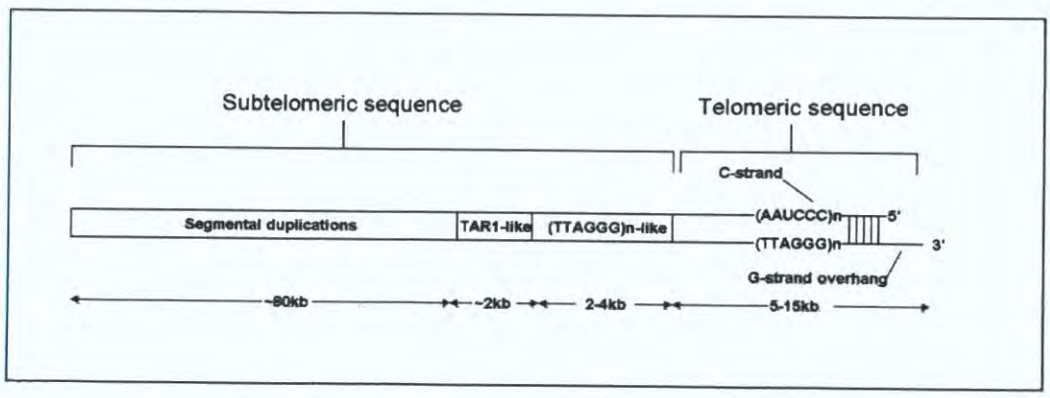

Figure 1.1 Structure of terminal 100kb of a human chromosome.
The contiguous stretch of TTAGGG repeats is interrupted by similar repeats which may differ on the basis of single nucleotide. This marks the beginning of the subtelomeric sequence. The remainder of the 100kb terminal chromosomal sequence and most of the subtelomere region is composed of segmental duplications. The T-loop is not shown in this diagram. Lengths shown are not to scale.

Up until recently telomeres were considered to be a region of constitutive heterochromatin that was transcriptionally inactive. However it has recently been shown that a heterogeneous non-coding RNA transcript called telomeric repeat RNA (TERRA) of 0.1-9kb long is transcribed using the telomeric C-strand and its adjoining subtelomeric DNA sequence as a template in a centromere-to-telomere direction (Azzalin et al., 2007). RNA polymerase II is responsible for TERRA transcription (Schoeftner and Blasco, 2008). There is evidence that TERRAs can interact with telomeric DNA to form an intermolecular G-quadruplex structure (Kimura et al., 2009). Non-coding RNAs (ncRNAs) can be responsible for mediating epigenetic changes at the gene loci. Two prominent examples are the HOTAIR and RepA ncRNAs which recruit complexes that induce repressive epigenetic changes (Rinn et al., 2007; Zhao et al., 2008). TERRAs have been found to localize with the sex chromosomes in embryonic stem cells (Zhang et al., 2009). Another study has highlighted that TERRA interacts with telomere-binding proteins and is essential for telomere heterochromatinisation (Deng et al., 2009). TERRA appears to be only briefly associated with telomeres during metaphase and it is therefore unlikely that it is a permanent constituent of the telomere complex (Azzalin et al., 2007; Schoeftner and Blasco, 2008).

1.1.2. Telomere proteins

Human telomeres are associated with a complex comprised of 6 proteins which is known as the shelterin or the telosome (Dubrana et al., 2001; de Lange, 2005). The six proteins of the shelterin complex (see Figure 1.2) include: Telomere repeat factors 1 and 2 (TRF1/2), Protection of Telomeres 1 (POT1), TRF1-interacting nuclear protein 2 (TIN2),

Repressor Activator protein 1 (RAP1) and the TIN2 and POT1 interacting protein (TPP1). TRF1 was the first component of shelterin to be identified on the basis of its affinity for telomere TTAGGG repeats (Chong et al., 1995; Broccoli et al., 1997). TRF1 and TRF2 are homologous proteins which both use their respective myb domains to bind telomeric DNA (Bilaud et al., 1997; Broccoli et al., 1997). TRF1 negatively regulates telomere length (van Steensel and de Lange, 1997; Li and de Lange, 2003). The TRF2 protein has a number of key roles. It prevents DNA repair being performed at the telomeres by inhibiting the activities of the ATM and ATR kinases (Karlseder et al., 2004; Denchi and de Lange, 2007). TRF2 is also involved in the formation of the T-loop to prevent the exposure of the 3' overhang (Stansel et al., 2001). TRF2 accomplishes this by facilitating ssDNA invasion of the duplex telomeric DNA sequence and then promoting a stalled homologous recombination event between the telomere repeats (Amiard et al., 2007; Poulet et al., 2009).

Although POT1 specifically binds to the 3'overhang sequence TAGGGTAG on the G-strand, evidence suggests that it is recruited to the telomere by TPP1 (Wang et al., 2007a). The 3'end of the telomere varies in terms of sequence whereas the 5'end of the telomere of 80% of chromosomes terminates with the sequence 3'-AATC-5' and POT1 is involved in generating this terminal sequence (Sfeir et al., 2005). The affinity of POT1 for the ssDNA overhang prevents the RPA protein from coating it, which is the first step in the ATR-initiated DNA damage pathway (Zou and Elledge, 2001; Denchi and de Lange, 2007). RAP1 interacts with TRF2 via its RCT domain (Li et al., 2000; Zhu et al., 2000a). RAP1 has BRCT and coiled-coil domains that recruit factors that negatively regulate telomere length (Li et al., 2003). It also interacts with a number of proteins

involved in the DNA damage response such as RAD50 and Mre11 (O'Connor et al., 2004).

TPP1 acts as a linker molecule that bridges the TRF1-TIN2 complex and POT1 and effectively recruits POT1 to the telomere, facilitating its binding to the 3' G-strand overhang (Houghtaling et al., 2004). TPP1 possesses a nuclear export signal and binds TIN2 and POT1 in cytoplasm and the nucleus (Chen et al., 2007). It is possible that it plays an instrumental role in at least partly contributing to shelterin assembly prior to its interaction with the telomere. TIN2 connects TRF1 and TRF2 and also bridges TPP1 and TRF1 (Li et al., 2000; Kim et al., 2004; Ye et al., 2004). TIN2 also interacts with tankyrase 1 which obstructs the ability of TRF1 to bind telomeres (Ye et al., 2004).

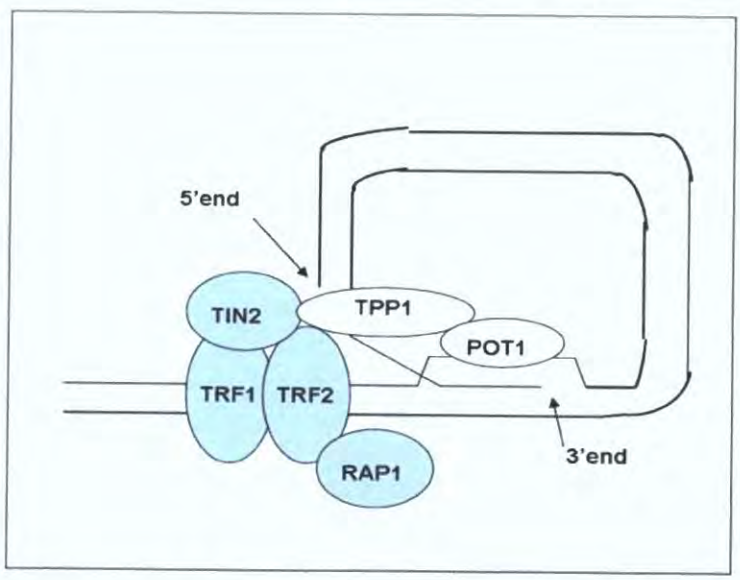

Figure 1.2. Structure of shelterin at the telomere terminus. The 3' ssDNA end of the telomere invades the dsDNA sequence to create a T-loop structure. This sequesters the 3'end of the telomere. The shelterin complex consists of 6 subunits which have been abbreviated as follows: POT1-protection of telomeres 1, TIN2-TRF-interacting nuclear protein 2, RAP1-repressor/activator protein 1, TRF1-Telomere repeat factor 1, TPP1 (formerly known as TINT1, PTOP or PIP1), TRF2-telomere repeat factor 2.

Aside from these shelterin components, there is a repertoire of other proteins that have been detected at the telomeres such as Mre11 and BLM helicase which assist in the homologous recombination required for T-loop formation and also tankyrases which regulate telomere length via the inhibition of TRF1 (de Lange, 2005). Most of these proteins are recruited by members of the shelterin complex (Songyang and Liu, 2006). Interestingly, telomeres actually recruit the homologous recombination machinery briefly during S-phase of the cell cycle possibly to generate the T-loop (Verdun and Karlseder, 2006). The highly compact nature of chromatin at the telomere can be inferred from a number of features. The length of linker DNA that connects one nucleosome with another is shorter than for the rest of the genome (Tommerup et al., 1994). Mammalian telomere nucleosomes typically exhibit a high frequency of repressive post-translational modifications that are characteristic of heterochromatin (de Lange et al., 1990; Makarov et al., 1993; García-Cao et al., 2004; Gonzalo et al., 2005, 2006). The high level of chromatin of chromatin condensation at the telomeres is at least partly responsible for the telomere position effect (Baur et al., 2001; Koering et al., 2002). Although it may be difficult to envisage shelterin proteins interacting with DNA in such a condensed state, it has been postulated that the altered topology of DNA on a nucleosomal surface may in fact make it more conducive to binding by these subunits (Pisano et al., 2008).

1.2. Telomerase

In 1972 James Watson proposed that 3'-5' replication of the lagging strand does not allow complete extension to the 3'end of the template (Watson, 1972). This is known as the 'end-replication' problem and is due to deletion of the most distal Okazaki fragment

that primes DNA synthesis. It was therefore hypothesised that a particular mechanism must counteract this gradual loss of telomere sequence that would be detrimental to long-term chromosomal viability. In a landmark paper in 1985 it was first reported that cell extracts of the ciliate *Tetrahymena* possess the functional capacity to extend telomeric DNA repeats in vitro (Greider and Blackburn, 1985). Based on this observation, a 'terminal telomere transferase' was postulated to be responsible for this activity and this nomenclature was later modified to 'telomerase'. Telomerase activity in human cells was detected a few years later (Morin, 1989). Telomerase activity has been observed in nearly all eukaryotic species as a telomere maintenance mechanism with exceptions such as a retrotransposition-based mechanism in *Drosophila* (Pardue and DeBaryshe, 2003). The human telomerase enzyme is a ribonucleoprotein complex and this architecture has been observed in most other eukaryotes to date, indicating a high degree of conservation.

1.2.1. Structure of telomerase

The active human telomerase holoenzyme that catalyzes nucleotide addition has been shown to date to be comprised of at least 4 components: human telomerase reverse transcriptase (hTERT), human telomerase RNA (hTR), telomerase Cajal body protein 1 (TCAB1; also known as WD repeat domain 79) and dyskerin (Cohen et al., 2007; Venteicher et al., 2009). However another study demonstrated that NHP2 and NOP10 are also subunits of the active telomerase holoenzyme (Fu and Collins, 2007). The hTERT gene was first cloned and sequenced over a decade ago (Meyerson et al., 1997; Nakamura et al., 1997). The hTERT catalytic subunit and the hTR RNA are sufficient to reconstitute telomerase activity in vitro (Weinrich et al., 1997; Beattie et al., 1998; Tesmer et al., 1999). The hTERT component is responsible for catalyzing nucleotide addition when

telomerase extends the 3' end of the telomeric DNA G-strand. High manganese ion levels (which usually encourage error-prone template-dependent DNA extension) promote catalytic nucleotide extension by hTERT in the absence of the hTR RNA (Lue et al., 2005). Determination of the primary sequence structure of hTERT revealed the presence of a number of motifs specific to the reverse transcriptase family which comprises the catalytic domain that is flanked on either side by a C-terminal extension (CTE) and an N-terminal extension as shown in Figure 1.3 (Lingner et al., 1997). A telomerase-specific region (called the T domain) is found only in the TERT sub-family and is located just adjacent to the RT domain (Kelleher et al., 2002). The NTE is necessary for telomerase catalytic activity (Xia et al., 2000; Armbruster et al., 2001). The CTE is not essential for catalytic activity but is required for telomerase processivity (Peng et al., 2001; Hossain et al., 2002; Huard et al., 2003).

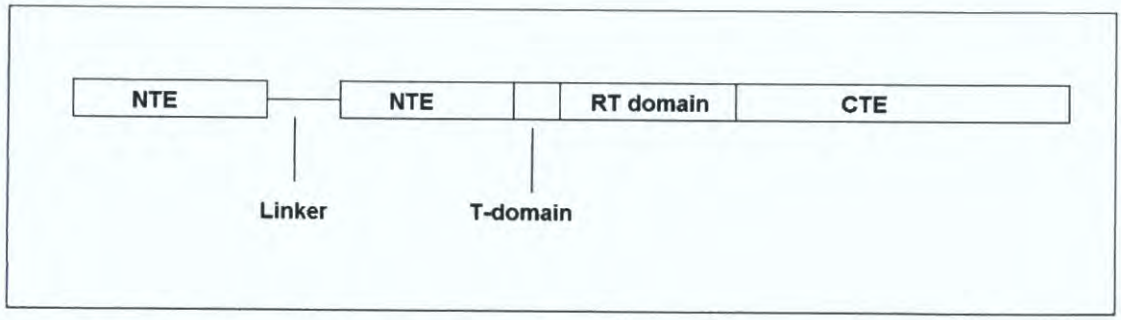

Figure 1.3. Schematic of hTERT protein. The Telomerase domain (T-domain is common to all known TERTs. Conserved sequences associated with catalytic nucleotide addition are found within the reverse transcriptase (RT) domain and these are found in all members of the reverse transcriptase family. The linker region is highly variable in sequence. CTE=C-terminal extension, NTE=N-terminal extension.

A region of hTERT encompassing a portion of the NTE and the adjoining T-domain are required for its interaction with hTR (Beattie et al., 2000; Bryan et al., 2000; Lai et al., 2001). hTERT has a conserved triad of aspartic acid residues that co-ordinate the

manganese ion that is essential to carry out catalytic nucleotide addition (Lingner et al., 1997). The crystal structure of the hTERT homolog in the potato beetle *Tribolium castaneum* has recently been solved and it confirmed that it exhibits the characteristic 'hands, palm and thumb' conformation that is associated with active nucleic acid polymerases (Gillis et al., 2008). Although telomerase activity is commonly associated with hTERT, it is known to have another function. hTERT was found to be involved in epithelial cell proliferation by activating the transcription of a number of genes that are required in the Wnt signaling pathway (Choi et al., 2008). This led to the discovery that it interacts with BRG1, a SWI/SNF-related ATP-dependent chromatin remodeling factor, and is localized at promoters of genes that are activated by Wnt signaling (Park et al., 2009). Wnt-dependent signaling is implicated in cell proliferation and so this may provide a means for hTERT upregulation to contribute to carcinogenesis (Reya and Clevers, 2005). In addition, hTERT has also been found to interact with an RNA processing endoribonuclease resulting in the formation of a ribonucleoprotein that exhibits RNA-dependent polymerase activity (Maida et al., 2009).

An RNA component was identified as a component of telomerase and was shown to function as a template to enable telomere extension (Greider and Blackburn, 1987; Yu et al., 1990). The hTR non-coding RNA component is transcribed by RNA polymerase II and the fully processed transcript contains 451 nucleotides (Feng et al., 1995). It can be approximately divided into 2 regions on a functional basis. The 5' end of the hTR transcript encompasses the pseudoknot and conserved region 4 and 5 (CR4/5) domains which are required for efficient telomerase activity (Tesmer et al., 1999). The hinge-hairpin-hinge ACA (H/ACA) box and the CR7 domain are found on the 3' section of the

hTR molecule and are necessary for the stabilisation of hTR. The H/ACA motif binds four proteins: Dyskerin, Nhp2, hNop10 and hGar1 (Mitchell et al., 1999; Dragon et al., 2000; Pogacić et al., 2000). These proteins interact with snoRNAs and are believed to play a role in stabilizing and processing the hTR RNA to produce a mature transcript that will associate with the telomerase complex. Due to repeated cycles of telomere extension by telomerase, it is important that the 3' telomere end sequence is elongated in a precise manner. hTR possesses a stem-loop structure (the P1b helix) that ensures telomerase does not extend the telomere beyond the 5' end of the template region (Tzfati et al., 2000; Chen and Greider, 2003).

The dyskerin protein (also known as H/ACA ribonucleoportein subunit 4, snoRNP protein DKC1, Nopp140-associated protein of 57kDa) is typically found as a component of nucleoprotein complexes. The dyskerin protein is typically localised to the nucleolus or to the Cajal body which is a subnuclear organelle where RNA modification and RNP assembly takes place (Heiss et al., 1999; Mitchell et al., 1999; Scherl et al., 2002). Dyskerin functions as a pseudouridine synthase due to its TruB domain which is homlogous to those found in the TruB proteins of bacteria (Koonin, 1996). In addition, dyskerin has a PUA domain that enables it to bind to RNA molecules and is therefore necessary for its inclusion in numerous RNP complexes (Pan et al., 2003).

Dyskerin plays a role in numerous biological activities, such as rRNA biogenesis as inferred from their association with the C/D and H/ACA snoRNAs (Filipowicz and Pogacic, 2002). Dyskerin was originally identified as a hTR-interacting protein (Dragon et al., 2000). Considering its role in RNP biogenesis, it was originally suspected that its

association with telomerase was based on its contribution to the assembly of the active telomerase holoenzyme, after which point, it would no longer be required. It has been discovered however that dyskerin remains associated with the active telomerase complex that catalyzes nucleotide addition (Cohen et al., 2007). It is possible that dyskerin carries out two roles in associating with telomerase: the first being to contribute to its assembly and the second perhaps being to stabilise the active telomerase holoenzyme through its interaction with the hTR RNA molecule. A 4th protein termed Telomere Cajal Body Protein 1 (TCAB1) has been recently found to be associated with the inactive and active forms of the telomerase complex (Venteicher et al., 2009). Although it is a component of the active enzyme complex it appears to be involved in telomerase assembly and localisation in two ways. First, it may facilitate additional assembly steps of the enzyme in Cajal bodies and secondly, it may also be responsible for inducing the translocation of telomerase from the cajal bodies to the telomeres.

A number of other proteins are known to associate with the telomerase complex and the majority interact specifically with the hTR and hTERT components. Telomerase-associated protein 1 (TEP1) interacts with hTR and hTERT via its N-terminal and C-terminal domains, but is not essential for telomerase activity (Harrington et al., 1997; Nakayama et al., 1997; Liu et al., 2000). The molecular chaperones p23 and p90 have been found to interact with the N-terminus of hTERT (Holt et al., 1999; Forsythe et al., 2001). The heterogeneous nuclear ribonucleoproteins C1, C2, A1 and UP1 associate with hTR in vivo (LaBranche et al., 1998; Ford et al., 2000; Fiset and Chabot, 2001). The hStau and L22 proteins also bind hTR (Ford et al., 2000; Le et al., 2000). The majority of

these proteins are probably implicated in the assembly and stability of telomerase as they have not been identified in purified fractions of the active telomerase complex.

1.2.2. Telomerase assembly

Telomerase assembly is a complex process involving the orchestration of a multitude of molecular events and appears to be controlled tightly in a temporal manner that is synchronised with cell cycle dynamics. Once transcribed, the hTR non-coding RNA component in particular must undergo a number of processing events. The stem-hinge-stem-ACA (H/ACA) motif is present in a large family of snoRNAs and is necessary for them to act as guides that induce the modification of rRNAs and snRNAs (Matera et al., 2007). The H/ACA motif is required for the co-transcriptional association of the dyskerin, hNOP10 and hGAR1 proteins with the hTR transcript (Darzacq et al., 2006). A biogenesis ('BIO') box is situated near the 3' terminus and is indispensable for hTR accumulation (Fu and Collins, 2006). The hTR RNA does not undergo the standard RNA polyadenylylation but is cleaved and modified with a 2, 2, 7-trimethylguanosine cap at the 5' end (Fu et al., 2006). Even though the hTERT catalytic subunit carries out the fundamental telomerase nucleotide addition, the hTR subunit RNP provides a scaffold upon which the telomerase holoenzyme is constructed.

hTR is directed to cajal bodies due to the presence of a CAB box which is present near its 3' terminus (Jády et al., 2004). Cajal bodies are subnuclear compartments associated with the nucleolus which are implicated in the assembly of RNP complexes (Matera and Shpargel, 2006). The hTR-associated RNP and hTERT remain separate throughout most of the duration of the cell cycle and only interact during the S phase of the cell cycle

(Tomlinson et al., 2006). The interaction of the hTR RNP and hTERT in vivo to form the telomerase holoenzyme complex requires the involvement of the chaperones hsp90 and SMN (Holt et al., 1999; Bachand et al., 2002). Evidence suggests that the Cajal bodies transport the fully assembled telomerase holoenzyme to the telomere (Jády et al., 2006; Tomlinson et al., 2006). Given that most of the components of the active telomerase complex are only a fraction of those that are present during its assembly, it remains to be determined how these proteins are dissociated prior to the maturation of the fully active telomerase complex.

1.2.3 Mechanism of telomerase reverse transcriptase activity

A model for the mechanism of telomerase was postulated as soon as evidence of the RNA component of telomerase emerged and has since proven to be remarkably prescient (Greider and Blackburn, 1989). To interact with the telomere, the hTR non-coding RNA base-pairs with the G-rich telomeric strand overhang and this ensures the correct positioning of telomerase to initiate the reaction (Figure 1.4). Another substrate interaction site for the telomerase enzyme has been deduced from experiments with hTERT orthologs which suggest that they also interact directly with the 5' terminus of the telomere (Hammond et al., 1997; Prescott and Blackburn, 1997). The hTERT subunit then uses the hTR nucleotides situated 5' of the region base-paired with the G-strand as a template for reverse transcription. Having extended the G-rich strand, the telomerase then disengages and interacts with the newly synthesised 3'terminus of the G-rich strand and begins another reaction cycle. This translocation step involves separating the DNA: RNA hybrid structure (in *Euplotes*, 8-10bp is disrupted) and the orientation of hTR relative to the telomerase complex has to be readjusted (Hammond et al., 1997; Kelleher et al.,

2002). Repeat-addition processivity is a term which describes the propensity of telomerase to continue translocating along a given *telomere* (Autexier and Lue, 2006) . The newly extended G-strand is then used as a template by the lagging strand synthesis machinery to convert most of the overhang into a dsDNA molecule. A number of studies suggest that the telomerase complex possesses nuclease activity but it is not yet known how this activity fits into the overall mechanism as currently understood (Melek et al., 1996; Lue and Peng, 1997; Huard and Autexier, 2004; Oulton and Harrington, 2004).

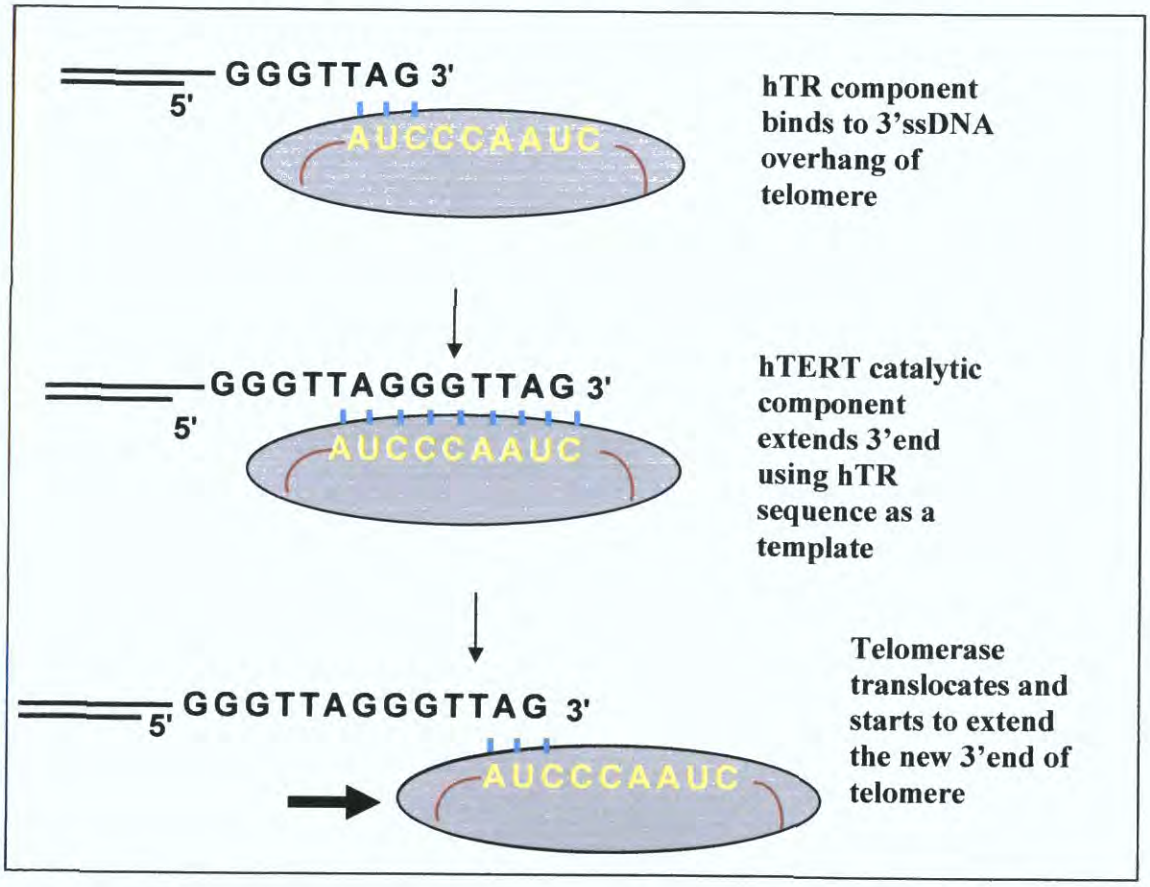

Figure 1.4. Mechanism of telomerase in extending telomere sequences. The hTR non-coding RNA is base-paired with the ssDNA G-strand. The 3'end is extended using the hTERT as a catalytic subunit for nucleotide addition and the hTR RNA as a template. Having extended the G-strand as much as the hTR RNA template will allow, telomerase disengages and interacts with new G-strand terminus to continue the process. The TCAB1 and dyskerin components are not shown.

1.3. Aberrant telomerase activation/repression and pathological consequences

1.3.1 Replicative senescence and crisis

Human primary cells typically lose 50-200bp of DNA with every cell division (Harley et al., 1990; Allsopp et al., 1992; Shay and Wright, 2000). This is caused partly by the end-replication problem and it is likely that other events occur which induce further recession of the terminal bases of telomeric DNA by nuclease-mediated degradation. One such mechanism for controlling telomere length involves the excision of the T-loop under circumstances in telomerase-positive cells when the telomere is excessively long, leading to the production of t-circles (Pickett et al., 2009). This event requires excision of the terminal bases of the lagging strand to permit the formation of a T-loop that is an essential feature of the telomeric architecture. The evidence for sequence-specific exonucleases acting on the telomeres can also be inferred from the fact that the 5' end of the C-strand always ends with the sequence 5'-ATTCAC-3' (Sfeir et al., 2005). Shortening of telomeres ultimately leads to cell cycle arrest or apoptosis (Harley et al., 1990; Hastie et al., 1990; Wellinger et al., 1996; Jacob et al., 2003).

Leonard Hayflick provided unequivocal proof that human foetal lung fibroblasts do not divide indefinitely in cell culture but undergo a finite number of divisions termed the 'Hayflick limit' (Hayflick and Moorhead, 1961). This demonstrated that cells reach a point where their reproductive potential is exhausted and cell division ceases but are still viable This particular stage is called 'replicative senescence' or mortality stage I (Wright and Shay, 1992). One characteristic of senescing cells is the presence of DNA damage

sites called telomere-induced foci where proteins such as NBS1 and 53BP1 are localised (d'Adda di Fagagna et al., 2003). The cessation of cell division is also accompanied by other physiological alterations such as morphological changes and the appearance of a β-galactosidase marker (Dimri et al., 1995). However, the presence of this marker is not specific to senescent cells and can be induced by other stresses (Severino et al., 2000). Another consequence of cellular senescence is the global heterochromatinisation of the genome in the form of condensed chromosomal structures known as senescence-associated heterochromatin foci (Di Micco et al., 2006; Zhang et al., 2007). This is presumed to repress genes that are implicated in cell cycle progression. The onset of replicative senescence is not linked to reduced telomere length per se but is more precisely correlated with the unsheathing of shelterin components associated with shorter telomere lengths. Overexpression of dominant-negative TRF2 in cells with telomeres of regular length induces replicative senescence through activation of the p53 and pRB pathways (Karlseder et al., 1999, 2004). The shelterin proteins TRF2 and POT1 inhibit the activity of the checkpoint proteins ATM and ATR (Karlseder et al., 2004; Denchi and de Lange, 2007). Loss of these proteins therefore leads to p53-mediated cell-cycle arrest. Although replicative senescence appears to be fundamentally linked to telomere attrition other stresses may induce this phenomenon (Wagner et al., 2009). Stress-induced senescence can be caused by a number of different types DNA damage or inappropriate growth stimulation. Mouse embryonic fibroblasts, despite their very long telomeres, cease dividing in cell culture after only 10 population doublings due to the high levels of oxygen (Itahana et al., 2004).

Simian virus 40 (SV40) is a polyomavirus that is frequently used to immortlaise human cells by enabling to escape the replicative senescence and crisis proliferative barriers as shown in Figure 1.5. It is widely considered that most developing tumour cells have similar growth kinetics. Disruption of tumour suppressor pathways enables cells to bypass the replicative senescence barrier and cell division continues until another barrier called crisis or mortality stage 2 is reached (Counter et al., 1992; Vaziri and Benchimol, 1999; Macera-Bloch et al., 2002). At this point telomeres have attained a length of 1-3kb. This consequently results in widespread cytogenetic abnormalities such as chromosomal end-to-end fusions, aneuploidy and regional chromosomal deletions (Chin et al., 1999; Artandi et al., 2000; O'Hagan et al., 2002). Crisis differs from replicative senescence in two respects: (1) there is widespread cell death (2) chromosomal abnormalities throughout the cell population become apparent. One study has revealed an important quantitative molecular distinction between cells undergoing senescence and crisis. 40% senescent BJ cells exhibit 'telomere associations' (where different chromosomal termini make contact, but do not form a covalent bond) whereas in crisis almost 100 % of cells do, with 60% of these possessing dicentric chromosomes (Zou et al., 2009). Such genomic instability induces multiple mutations, many of which are detrimental to cell viability. The increased mutation rate may also help to accelerate the successive acquisition of characteristics that facilitate tumour development (DePinho and Wong, 2003). Some of these mutations enable cells to stabilise their telomere lengths through the activation of a telomere maintenance mechanism (TMM) (Counter et al., 1994). Cancer cells have to overcome both replicative senescence and crisis barriers and TMM-conferred immortality is considered one of the six molecular hallmarks that define a

fully-fledged tumour (Hanahan and Weinberg, 2000). Two such mechanisms have been identified to date: telomerase activation and ALT.

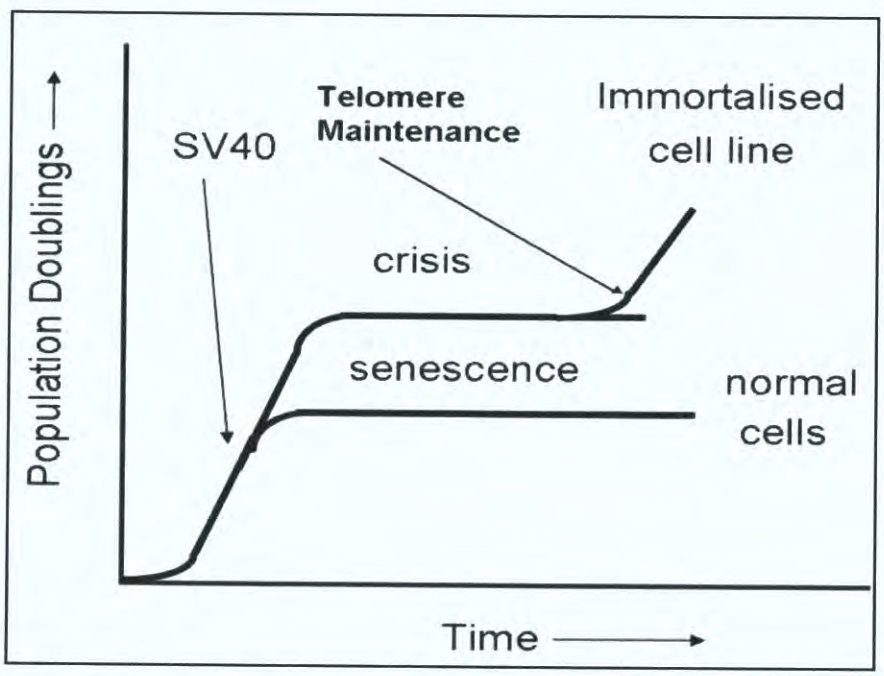

Figure 1.5. Pathway to cellular immortalization.
Normal somatic cells divide until their telomere lengths reach ~4-6kb which usually triggers replicative senescence. At this point proliferation ceases unless an event such as SV40 transfection occurs when the large T antigen inhibits the tumour suppressors p53 and pRb. This allows the cell to overcome replicative senescence until telomere DNA reaches a critical length (~1-3kb). This causes genetic instability incompatible with cell viability (crisis). Tumour and immortalised cells can continue to divide beyond this point if they activate/have already activated a TMM.

1.3.2. Telomerase activation

The importance of telomerase activity in cancer is highlighted by the fact that it is present in 85-90% of cancers (Kim et al., 1994; Shay and Bacchetti, 1997). In humans, telomerase activity is present in germinal stem cells, adult stem cells and activated B-cells but is silenced in somatic cells (Blasco et al., 1996; Roth et al., 2003). The upregulation of telomerase in cancer cells involves switching on hTERT gene expression

and/or increasing the copy number of the hTERT gene locus (Cao et al., 2008). Although telomerase is not found in most somatic cells, hTERT expression occurs transiently during the S phase of cell division, but is not sufficient to compensate for telomere loss due to cell division (Masutomi et al., 2003).

hTERT is the most commonly upregulated gene associated with cancer (Cortez-Gonzalez and Zanetti, 2007). Because of this it is widely considered to be a potential therapeutic target. A number of chemical telomerase inhibitors have been developed and immunotherapy strategies devised to obstruct its activity. However under clinical circumstances telomerase needs to be repressed by 60% for up to 250 cell divisions which may induce side effects (Blagoev, 2009). Despite its prevalence, telomerase is not defined as an oncogene in view of the fact that it facilitates but does not actively stimulate continuous cell proliferation (Harley, 2002). This perspective may have to be revised considering that evidence is being accumulated that telomerase can encourage tumorigenesis by mechanisms that are independent of its capacity to catalyze nucleotide extension.

A number of studies have shown that hTERT expression can counteract apoptosis (Cao et al., 2002; Del Bufalo et al., 2005). hTERT has been shown to prevent cell death in neuronal cells that are subjected to stresses such as the presence of β-amyloid plaques or ischaemia (Zhu et al., 2000b; Kang et al., 2004). Survivin is a protein that confers resistance to apoptosis and is upregulated in tumour cells (Ambrosini et al., 1997). The presence of hTERT is necessary for the expression of survivin (Yuan et al., 2009). hTERT can also stimulate stem cell proliferation in hair follicles and is associated with

the reprogramming gene expression to accomplish this (Sarin et al., 2005; Choi et al., 2008). The hTR component of telomerase has been shown to promote evasion of DNA damage checkpoints (Blasco et al., 1996; Kedde et al., 2006).

One important aspect of telomerase activation is how it influences the course of tumour development and therefore its clinical ramifications. Telomerase-positive tumours appear to have more vigorous growth properties than their telomerase-negative counterparts. Only 29.5% of benign non-metastatic cancers exhibit telomerase activity, in contrast to 84% of malignant cancers (Shay and Wright, 2007). A number of studies collectively suggest that the presence of telomerase activity in tumours is typically associated with enhanced tumoral severity and increased frequency of recurrence. Patients suffering from neuroblastoma lacking telomerase activity have survival rates of 93% whereas those which had telomerase had survival rates of 34% (Poremba et al., 2002). In hepatoblastomas, it was found that in telomerase-positive tumours there is 60% chance of recurrence within 10 years, compared to 10% for telomerase-negative tumours. Lack of telomerase activity in ependymomal tumours correlates with an increased probability of 5-year survival compared to telomerase-positive equivalents (Tabori et al., 2006). Mouse cell lines that use the ALT mechanism are less able to induce metastases than their telomerase-positive counterparts (Chang et al., 2003).

1.3.3. ALT

Although the majority of immortal cancer cells employ telomerase activation as a TMM, the remaining 10-15% avail of a mechanism called the alternative lengthening of telomeres (Bryan et al., 1995, 1997). The ablation of telomerase activity in yeast

generates 2 classes of survivors: type I and type II. Type II exhibit very long telomeres due to a recombination-based system of extension (Lundblad and Blackburn, 1993). It was believed that a similar mechanism was responsible for telomere maintenance in telomerase-negative cancer cells in humans. It was later confirmed that homologous recombination of telomeric DNA sequences occurs in cells employing ALT (Dunham et al., 2000). Aside from the absence of telomerase activity, ALT-positive cells have a number of other characteristics that are used to distinguish them from telomerase-positive counterparts. One of the most apparent traits is the extent of the heterogeneity in telomere length that ranges from 2-50kb in length (Henson et al., 2002). This is perhaps due to the possibility that telomeric DNA recombination is less regulated than telomerase activity.

Another indicator of the ALT mechanism is the relatively high quantity of extrachromosomal telomeric repeat DNA that assumes a linear or circular form (Ogino et al., 1998; Tokutake et al., 1998; Wang et al., 2004a). It is likely that that the t-circles are generated due to the appearance of very long telomeres in ALT-positive cells. Another characteristic of the ALT phenotype is the presence of ALT-associated PML bodies (APBs). These are composed of extrachromosomal telomeric DNA, shelterin proteins and proteins associated with DNA repair and recombination such as Mre11 and NBS1 (Yeager et al., 1999; Nabetani et al., 2004). It has been shown that APBs are responsible for bringing 2-5 chromosomal termini into close proximity with each other to facilitate recombination (Draskovic et al., 2009).

The precise mechanism of telomere extension in ALT cells has not yet been fully elucidated, but the details of the underlying pathways are beginning to emerge. It is

generally accepted that recombination is necessary, with its frequency being increased at the telomeres but not elsewhere in the genome (Bechter et al., 2003). Evidence has been provided that telomeric recombination occurring in ALT cells can happen due to exchange of DNA within the same chromosome due to intratelomeric recombination (Muntoni et al., 2009). Telomeres in ALT-positive cells undergo recombination with their sister chromatid counterparts during replication, but these events do not result in a net gain or loss of sequence (Bailey et al., 2004). In this context recent theoretical modeling has demonstrated that sister chromatid exchange as the sole ALT mechanism can only be viable if longer chromatids are preferentially co-segregated into the same cell during anaphase (Blagoev and Goodwin, 2008).

The most popular model for telomere recombination is that of the break-induced replication model (Royle et al., 2009). In this model the G-strand of one telomere pairs with the C-strand of another telomere and displaces its counterpart C-strand. In so doing it enables it to be extended by a DNA polymerase using its new partner on the C-strand as a template. In this way ALT can ensure a net gain of telomeric DNA sequence that counteracts the loss of these sequences due to cell division. It is also possible that recombination between telomeres and extrachromosomal telomeric repeat DNA also occurs, although this has not yet been experimentally verified. Just as pRb can indirectly downregulate telomerase activity, another member of the Rb protein family, p130, can repress ALT (Kong et al., 2006). One cell line has been discovered that maintains its telomeres via recombination but does not possess other hallmarks of the ALT mechanism (Cerone et al., 2005).

1.3.4. Telomerase and Ageing

The gradual depletion of telomeres in the absence of a TMM is correlated with ageing (Harley et al., 1990). One of the outstanding issues concerning the relationship between telomere length and ageing is whether or not telomere exhaustion is partly a cause or effect of the complex phenotype that ageing represents. The number of cell divisions undertaken is linked to age and negatively impacts on telomere length (Harley et al., 1990). In addition, the high GC content of telomeres makes them vulnerable to oxidative damage that accumulates with age (Saretzki and Von Zglinicki, 2002). Therefore, telomere length could be regarded as a marker of ageing that serves as a 'mitotic clock'. Studies in mice have revealed that at least some of the symptoms of ageing can be attributed to shorter telomere lengths. Mice bred to have short telomeres exhibit a more rapid decline in tissues of the hematopoetic and immune systems, where extensive cell recycling occurs (Armanios et al., 2009). Further evidence that telomere attrition makes at least a partial contribution to ageing was provided when it was demonstrated that the artificial induction of telomere dysfunction in mice results in the expression of 4 protein markers such as CRAMP and stathimin whose homologs are overexpressed during ageing in humans (Jiang et al., 2008).

The 'disposable soma' hypothesis states any energy invested in the upkeep of the body or the 'soma' is at the expense of energy devoted towards reproduction and the perpetuation of the genetic material. Telomere lengths have been set by evolutionary factors to ensure a lifespan that satisfies reproductive requirements and offspring nurturance. An additional purpose of the evolutionary calibration of the telomere length is to obstruct indefinite cell

proliferation that would increase the probability of mutations occurring that contribute to tumorigenesis (Vogelstein and Kinzler, 1993). In somatic cells, therefore, replicative senescence constitutes a powerful tumour suppressor mechanism (Campisi, 2001). In support of this idea, mice engineered to have constitutive telomerase activity have enhanced wound-healing capacity but there is a higher incidence of tumours (González-Suárez et al., 2001). When such mice are bred with those overexpressing key tumour suppressors the offspring overcome this problem and have a longer lifespan without the associated side-effects (Blasco, 2007).

The replenishment of cells in a particular organ or tissue is necessary to sustain its functionality and is dependent on the adult stem division dynamics. The precise mechanism of how reduced telomere lengths can cause the ageing of a particular tissue is not yet fully understood. Adult stem cells are situated in a distinct site within a tissue termed the stem cell niche (Fuchs et al., 2004a). Adult stem cells undergo asymmetric cell division that typically gives rise to one daughter stem cell and one committed progenitor cell. Although telomerase activity is expressed in adult stem cells it is not sufficient to compensate for telomere attrition (Ramirez et al., 1997; Yui et al., 1998). One theory of ageing proposes that the committed progenitor is unable to leave the niche when its telomeres are sufficiently shortened (Blasco, 2007). This mitigates the replenishment of cells that have undergone replicative senescence or apoptosis with the result that the functional efficacy of the tissue deteriorates over time. Typically telomere losses of approximately 1kb/year occur in the first 4 years of life, with degradation rates becoming more gradual thereafter (Frenck et al., 1998). A number of studies have identified an inverse correlation between telomere length and mortality (Cawthon et al.,

2003; Bischoff et al., 2005; Njajou et al., 2007). One study found that centenarians of Ashkenazi Jewish descent and their offspring showed superior telomere maintenance properties to their controls (Atzmon et al., 2009). The centenarians had a higher prevalence of particular synonymous intronic mutations in the hTERT gene. This suggests that SNPs in components of the telomerase enzyme can modulate the rate of ageing.

Highly proliferative cells such as stem cells, progenitor cells, lymphocytes and skin keratinocytes maintain high levels of telomerase activity (Flores et al., 2006). Shortened telomeres have been linked with a number of geriatric disorders. Although cancers must upregulate telomerase its absence until that point predisposes one to this disease due to telomere shortening. A number of patient studies have correlated telomere shortening with the incidence of diabetes (Jeanclos et al., 1998; Adaikalakoteswari et al., 2005; Sampson et al., 2006). Reduced telomere lengths are associated with a number of gastrointestinal disorders such as Barrett's Esophagus and ulcerative colitis (O'Sullivan et al., 2002; Finley et al., 2006). Non-alcoholic fatty liver disease is linked to increasing age (Miyaaki et al., 2008; Frith et al., 2009). Shorter telomere lengths have also been correlated with this disorder. Rheumatoid arthritis is usually a geriatric disease and is characterized by tissue damage at joints due to excessive inflammation, which is at least partly caused by T-cell dysfunction (Goronzy and Weyand, 2004). Decreased telomerase activity results in shorter telomeres in naïve and memory T-cells which enhances their susceptibility to apoptosis in this condition (Fujii et al., 2009). Shorter telomere lengths are also associated with the incidence of Alzheimer's disease (Panossian et al., 2003). Bone mineral density declines as a function of age and causes an increase in the

frequency of fractures occurring. Telomere length is significantly correlated with bone mineral density (Bekaert et al., 2005). One study showed a correlation between telomere length and the frequency of inflammation as determined by the presence of C-reactive protein (Aviv et al., 2006). Short telomeres have been characterized in a number of haematological disorders. Telomere lengths are more reduced in cardiac cells of heart biopsies from heart failure patients compared to non-diseased controls (Chimenti et al., 2003; Oh et al., 2003). A mutation in the hTERT promoter represents a significant risk factor for coronary artery disease (Matsubara et al., 2006). Shorter leucocyte telomere lengths are a risk factor for atherosclerosis and a high percentage of cells in the atherosclerotic plaque itself are senescent (Samani et al., 2001; Minamino et al., 2002). Higher social status is correlated with longer telomeres (Cherkas et al., 2006).

1.3.5. Diseases of telomerase deficiency

Dyskeratosis congenita (DC)

Although described nearly a century ago, Dyskeratosis Congenita was characterized as a disease in 1975 on the basis of 3 key symptoms: (1) Oral leukoplakia (2) Dermal hyperpigmentation and (3) nail dystrophy (Sirinavin and Trowbridge, 1975). It is a very heterogeneous disorder also accompanied by a range of other symptoms in a number of other tissues where more frequent cell recycling is required. The prevalence of this disorder is 1 in a million and the median age of death is 16 years (Drachtman and Alter, 1995). It would seem counterintuitive that DC can result in a high cancer incidence considering telomerase upregulation is a typical characteristic of most tumours. However, up to 10% patients with DC succumb to cancer.

DC has three familial modes of inheritance: autosomal recessive, autosomal dominant and X-linked recessive. 50% of DC cases are due to mutations in the genes which code for 3 known components of the active telomerase complex: DKC1 (dyskerin), hTERT and TERC. The gene encoding dyskerin, *DKC1*, was one of the first shown to cause DC in an X-linked manner (Knight et al., 1996; Heiss et al., 1999). The autosomal recessive version of DC is caused by mutations in the *NHP2* and *NOP10* genes which are involved in stabilization and processing of the hTR non-coding RNA (Vulliamy et al., 2008; Walne et al., 2008). Autosomal dominant inheritance occurs due to a mutation in the catalytic domain of the hTERT allele indicating that just one functioning allele is insufficient for telomere maintenance (Armanios et al., 2007). Mutations in *TINF2*, a gene which encodes the shelterin protein TIN2, are also correlated with DC (Savage et al., 2008).

Hoyeraal-Hreidarsson syndrome (HH)

This is a highly systemic disorder, affecting multiple tissues and presents a number of symptoms also observed in DC which can occasionally obfuscate proper distinction between both diseases in a clinical setting (Knight et al., 1999). This illness could be essentially considered a more extreme variant of DC with more severe symptoms and shorter lifespan. Patients with HH are generally diagnosed at an earlier age and suffer from retarded post-natal and pre-natal growth. So far mutations associated with this syndrome have been identified in the *TINF2* and *DKC1* loci (Knight et al., 1996; Walne et al., 2008). In some cases of HH a patient is homozygous for a mutant allele of hTERT for which both of the parents were heterozygous but did not exhibit as severe a clinical phenotype (Marrone et al., 2007). This notion is supported by the fact that it is caused by

homozygous mutations that inhibit hTERT and TERC functionality, thereby completely ablating any telomerase activity.

Idiopathic Pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is a respiratory disease that is the most common of the interstitial idiopathic pneumonias and is a consequence of defective gas exchange resulting from destruction of lung tissue (Gross and Hunninghake, 2001). The incidence of IPF ranges from 4/100,000 in the 18-34 years old age group to 277/100,000 in the over 50 age bracket (Garcia et al., 2007). The disease has both genetic and sporadic variants with 20% of those afflicted reporting a family history of the disorder (Loyd, 2003). The prognosis for IPF is fatal with death usually occurring 2-5 years after diagnosis (Khalil and O'Connor, 2004). Shortened telomeres have been observed in alveolar and leucocyte cells in both forms of IPF (Cronkhite et al., 2008). Mutations have been identified in the telomerase components hTERT and hTR in 8-12% of those affected by the genetic version of IPF (Armanios et al., 2007; Tsakiri et al., 2007). The mutations in these loci are different from those that are responsible for DC and it is therefore possible that they reduce telomerase activity to a lesser extent, resulting in a less deleterious disease phenotype.

Haematological disorders

Although aplastic anaemia (AA) is a condition in its own right, AA can also be itself as a clinical manifestation of DC. Like IPF, AA has relatively low rate of incidence, with 2-5 million cases being diagnosed each year (Kirwan et al., 2008). A notable physical characteristic of this disease is the hollow appearance of the bone marrow. This leads to a

lack of red blood cells, white blood cells and platelets. As a result, affected patients exhibit symptoms such as fatigue and increased susceptibility to infections. 3% of patients that present with this condition have genetic mutations in hTR and hTERT genes (Feldser and Greider, 2007; Yamaguchi et al., 2003, 2005). Myelodysplastic syndrome (MDS) is a collective term that encompasses a range of haematological disorders which all have defective blood cell generation. MDS typically only affects those cells of the myeloid lineage. An enhanced degree of telomere shortening in granulocytes and monocytes has been detected in this disorder (Ball et al., 1998; Rigolin et al., 2004).

1.4. Regulation of telomerase

Although not expressed in somatic cells which constitute the majority of the overall cell population of the human body, telomerase is subject to regulation by a plethora of diverse mechanisms. The complexity of its regulation reflects the importance of telomere length as a molecular chronometer that senses the 'age' of the cell which is a key parameter in determining commitment to differentiation or further proliferation. Telomerase regulation can be divided from a temporal perspective into 2 key phases: (1) Regulation of the subunits that comprise the active telomerase complex (2) Regulation of the fully active telomerase complex. For (1), most attention will be devoted to regulation of the hTERT subunit which is regulated predominantly at the transcriptional level by a number of activators and repressors.

1.4.1. Regulation of telomerase components other than hTERT

Increased levels of the hTR non-coding RNA component have been detected in cancer cells (Heine et al., 1998; Kyo et al., 1999a). In addition, the hTR gene chromatin environment in telomerase-positive cells has histone modifications associated with transcriptional activation in contrast to telomerase-negative cells (Atkinson et al., 2005). However, hTR is ubiquitously expressed in somatic cells that are telomerase-negative (Tesmer et al., 1999). Higher levels of dyskerin in tumours have been correlated with increased telomerase activity (Montanaro et al., 2006, 2008). The dyskerin component of telomerase is also ubiquitously expressed and therefore is not likely to be a significant contributor to hTERT regulation (Heiss et al., 2000). Another study compared expression of telomerase subunits in normal and cancer cells and found only hTERT to be upregulated in cancer (Chang et al., 2002). The number of telomerase complexes in a cell has been estimated to be ~100 (Cohen et al., 2007). If the number of hTR transcripts or dyskerin proteins exceeds this, it would not result in an increase in telomerase activity. It is therefore unlikely that their levels are critical determinants in telomerase regulation. Furthermore, ectopic expression of hTERT alone is sufficient to activate telomerase in some cell types (Bodnar et al., 1998; Counter et al., 1998; Yang et al., 1999). It is therefore currently widely acknowledged that the regulation (esp. at the transcriptional level) of the hTERT catalytic subunit is the key rate-limiting component in the overall control of telomerase activity. For this reason, the discussion of telomerase regulation will be mainly focussed on the regulation of hTERT. In particular, a variety of transcription factors (TFs) which control hTERT expression have been identified (see Table 1.1).

Table 1.1. List of transcription factors (TFs) that regulate the hTERT promoter

Activators	Repressors
Sp1	p53
c-Myc	BRCA1
USF1/2	WT1
Ets2	E2F
ER81	Sp3
Estrogen receptor	Ap-1
PAX5	SMAD3
PAX8	Cbfa1
NFAT	SIP1
AP-2	NFX1-91
HIF-1	

1.4.2. hTERT transcriptional activators

The hTERT promoter was cloned by a number of groups and led to the identification of a number of transcription factor binding sites (see Figure 1.6) that were experimentally verified (Cong et al., 1999; Horikawa et al., 1999; Takakura et al., 1999; Yi et al., 1999). Specifity protein 1 (Sp1) is a ubiquitously expressed C_2H_2-type zinc-finger transcription factor that binds to a GC-box cognate site and is required for cell growth and development (Zhao and Meng, 2005). Initial sequence analysis of the hTERT gene revealed the presence of 5 GC-boxes located in the core promoter region between the 2 E-boxes. A number of studies confirmed that Sp1 postively modulates hTERT gene expression (Cong and Bacchetti, 2000; Kyo et al., 2000). Sp1 activates transcription in one way by recruiting components of the basal transcription machinery such as TBP-associated factors (TAFs) (Emili et al., 1994; Gill et al., 1994). The hTERT promoter lacks a TATA box to recruit TATA-binding protein and so the number and density of GC boxes may be necessary to recruit the basal transcriptional machinery to the hTERT promoter.

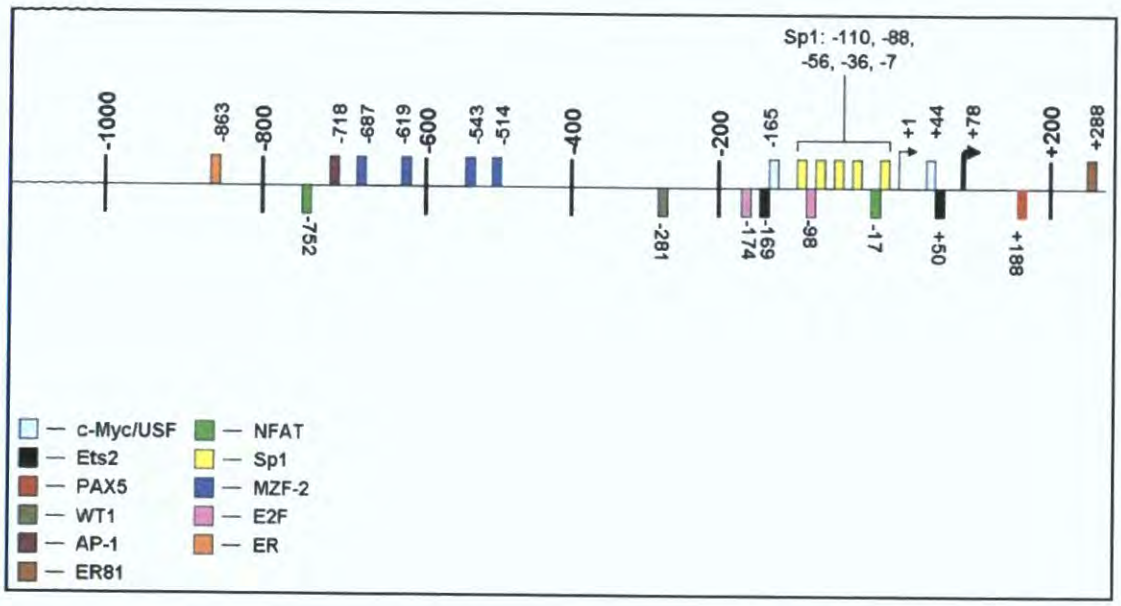

Figure 1.6. Anatomy of the hTERT promoter.
'+1' denotes the first transcribed nucleotide as previously described (Takakura et al., 1999). '+78' denotes the first nucleotide of the translation initation codon. Numbers shown refer to the first 5' nucleotide of the transcription factor binding sites (in the direction of transcription). TFs whose binding sites on the hTERT promoter have not yet been precisely determined are not displayed.

c-Myc is a highly characterised proto-oncogenic transcription factor which is overexpressed in a wide variety of cancers. Under normal circumstances, c-Myc acts as a master regulator that switches on the expression of genes that are involved in cell proliferation and it is indispensable for mammalian embryonic development (Grandori et al., 2000). Myc possesses a basic helix-loop-helix zipper domain that enables it to interact with E-boxes to activate transcription that forms a heterodimer with the Max homolog and binds to the E-box sequence CACGTG usually located upstream of genes to activate transcription. Initial sequence analyses revealed the presence of two such sites in the hTERT promoter, beginning at nucleotides -165 and +44 in relation to the transcription

start site (Cong et al., 1999; Horikawa et al., 2002). In accordance with this observation, a number of studies have reported that c-Myc activates hTERT expression (Greenberg et al., 1999; Wu et al., 1999; Gil et al., 2005; Benanti et al., 2007) . c-Myc binds both E-boxes with equal affinity and induces DNA bending that may facilitate the recruitment of other TFs/coactivators to the hTERT promoter (Lebel et al., 2007). c-Myc and Sp1 act in a cooperative manner to stimulate hTERT gene transcription (Takakura et al., 1999; Kyo et al., 2000) . In Human Leukaemia 60 (HL60) cells, differentiation is preceded by a shutdown in telomerase activity that that is at least partly caused by the substitution of the c-Myc: Max heterodimer at the hTERT promoter with the Mad:Max repressive complex (Xu et al., 2001; Jiang et al., 2006a) . The more notable frequency of telomerase activity attributed to late stage tumours is correlated with enhanced levels of c-Myc (Greider, 1998; Sedivy, 1998). The hTERT gene is located on the chromosome proband 5p15.33 which is the closest to the telomere and may have regulatory implications (Meyerson et al., 1997; Bryce et al., 2000). One study has proposed that a shorter telomere sequence results in genomic instability that disrupts the repressive chromatin environment of the hTERT promoter and permits c-Myc binding (Bazarov et al., 2009).

Upstream stimulating factors 1 and 2 (USF1 and 2) are both basic helix-loop-helix zipper proteins that interact with the consensus sequence CANGTG (Gregor et al., 1990; Sirito et al., 1994). USF1 and 2 are both ubiquitously expressed and are known to be involved in the cell cycle progression in addition to other other cellular processes (Corre and Galibert, 2005). USF1 and USF2 also interact with E-boxes in the hTERT gene as a

heterodimeric complex and increase its rate of transcription in telomerase-positive cells (Yago et al., 2002; Goueli and Janknecht, 2003; Jiang et al., 2010).

Telomerase is subject to regulation by a number of hormones and cytokines. The E26 transformation specific (Ets) TFs act downstream of the EGF receptor and are implicated in processes such as cell proliferation and differentiation by coupling hormonal stimulation to gene expression. Members of the Ets family of transcription factors can act as repressors or activators and are all characterized by a conserved 85 amino acid sequence called the Ets domain. The Ets transcription factors recognize the consensus sequence GGAA/T and overexpression of Ets1 and Ets2 is generally associated with the promotion of tumorigencicty and cell proliferation. Ets2 interacts with two GGAA boxes adjacent to the 2 E-boxes on the hTERT promoter (Maida et al., 2002; Xiao et al., 2002; Hsu et al., 2006). One study confirmed that Ets2 plays a very important role in contributing to hTERT transcriptional activity in MCF-7 cell line, but there was no evidence that the Ets1 homolog regulates hTERT transcription (Xu et al., 2008a).

ER81 (also known as ETV1) is a TF that belongs to the PEA3 subfamily of the Ets TF family and is activated by Her2/Neu, Ras and Raf to stimulate transcription from the hTERT promoter (Goueli and Janknecht, 2004). One study has shown that activation of the Her2/Neu receptor or overexpression of Ras and Raf downstream components stimulates the ERK MAP kinase pathway, resulting in hTERT transcription. Experimental evidence indicates that ER81 upregulates hTERT transcription in breast carcinoma cells (Vageli et al., 2009). Ewing's sarcoma is a malignant tumour that arises in the bone or soft tissue (Iwamoto, 2007). The underlying aetiology of Ewing's sarcoma

is due to the fusion of the *EWS* gene to one of five members of the Ets gene family: *Fli1*, *ERG*, *ER81*, *PEA3* and *FEV*. The *EWS/E1AF* and *EWS/FLI1* gene fusions can induce an upregulation in hTERT mRNA levels, highlighting one means via which these fusion proteins facilitate tumorigenesis in this disorder (Fuchs et al., 2004b; Shindoh et al., 2004).

Cells of the female reproductive system, especially those comprising the endometrium, undergo extensive recycling and telomerase activity is necessary to compensate for telomere loss to sustain this high rate of cell division. Estrogen induces telomerase activity in a number of cell types that express the estrogen receptor (Misiti et al., 2000; Imanishi et al., 2005; Doshida et al., 2006; Sarkar et al., 2006; Calado et al., 2009;). Tamoxifen, a competitive inhibitor of the estrogen receptor downregulates telomerase activity in breast cancer cell lines (Aldous et al., 1999; Wang et al., 2002). Bioinformatic analysis of the hTERT promoter sequence revealed the presence of two estrogen response elements at -950 and -2754 relative to the translation start codon (Cong et al., 1999; Takakura et al., 1999) . Both binding sites are necessary for estrogen-stimulated hTERT transcription (Kyo et al., 1999b; Misiti et al., 2000).

The Paired Box (PAX) protein family consists of a number of transcription factors with a characteristic DNA-binding motif and play an important role in tissue-specific patterning (Underhill, 2000). PAX8 is one such protein which is involved in the development of several tissues derived from the germ layers of the mammal embryo (Mansouri et al., 1999). Four binding sites for the PAX8 gene have been identified in the hTERT promoter, three of which are located in the hTERT core promoter region (Chen et al.,

2008) . PAX 8 binds to the hTERT promoter in vitro and is capable of activating transcription at this site when overexpressed specifically in glioma-derived cell lines (Chen et al., 2008). PAX5 is another member of this family and its expression is found in developing B-cells prior to undergoing terminal differentiation into plasma cells (Barberis et al., 1990; Nutt et al., 1997). PAX5 was found to be implicated in hTERT transcriptional regulation in these cells by interacting with 2 binding sites downstream of the transcription start site and can activate hTERT expression in telomerase-negative fibroblasts (Bougel et al., 2010).

NFAT proteins are a family of TFs which bind to the particular consensus sequence GGAAA and activate genes that contribute to the activation of T-cells in the immune response (Rao et al., 1997). There are 4 binding sites for the NFAT TF on the hTERT promoter located at -1575, -1225, -1200 and -40, with the one located at -40 is required for maximal hTERT transcription in Jurkat and MCF-7 cells (Chebel et al., 2009). The Activating Enhancer-binding protein (AP-2 family) of transcription factors consists of 5 different isoforms in humans that assemble homo- and heterodimers and bind to the GC-rich consensus sequence. AP-2β contributes to retinoic acid-based development, especially in the neuroectoderm (Moser et al., 1997). It has been discovered that one member of the AP-2 family, AP-2β binds to the hTERT promoter and activates hTERT transcription in lung cancer cells (Deng et al., 2007). Grainyhead-like2 (GRHL2; also known as BOM,) is homologous to the grainyhead proteins in drosophila that participate in dorsoventral patterning and are currently known to be essential for ear development in humans (Wilanowski et al., 2002). GRHL2 was isolated as positive TF regulator of the hTERT locus in oral squamous carcinoma cells using a promoter magnetic precipitation

assay (Kang et al., 2009). Its binding site on the promoter has been approximately estimated to lie somewhere between nucleotides -49 to +5.

Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcription factor whose subunit resists degradation under low O_2 conditions. HIF activates a suite of genes that counteract the stress imposed upon the cells during such circumstances. There are two HIF-1 response elements located on the hTERT promoter from -165 to -158 and from +44 to +51 (Nishi et al., 2004). HIF-1 activates hTERT expression in choriocarcinomas and cervical cancer cells (Nishi et al., 2004; Yatabe et al., 2004). One means by which HIF-1 contributes to hTERT activation is through recruitment of TFIIB to the hTERT promoter and thereby prompting assembly of the pretranscriptional complex (Anderson et al., 2006).

Many viruses stimulate proliferation of the host-infected cells and the upregulation of telomerase is required to facilitate further cell division. Many viruses have therefore evolved the requisite machinery to stimulate telomerase activity. Human T-cell leukaemia virus type 1 (HTLV-1) is a notable example of one such virus. The HTLV-1 bZIP (HBZ) transcription factor is expressed by this virus and interacts with JunD to from a complex which in turn interacts with Sp1 in binding the hTERT promoter to drive hTERT transcription during the late stage of the infection process (Kuhlmann et al., 2007). The HTLV-1 Tax protein also positively regulates hTERT gene expression (Sinha-Datta et al., 2004). A number of studies have demonstrated that the human papilloma virus 16 E6 protein upregulates hTERT transcription (Gewin and Galloway, 2001; Oh et al., 2001; Veldman et al., 2001). Human Cytomegalovirus immediate early antigen 72 can transactivate the hTERT gene via relief of histone deacetylase occupancy

at the promoter (Stråål et al., 2009). The latency-associated nuclear antigen (LANA) expressed by Kaposi's sarcoma-associated herpesvirus stimulates hTERT transcription (Knight et al., 2001; Verma et al., 2004). It acts on the promoter region from -130 to +5 and its ability to transcriptionally activate the hTERT gene is Sp1-dependent.

1.4.3. hTERT transcriptional repressors

p53 is a multifunctional tumour suppressor protein that carries out tasks such as controlling gene expression (it both activates and represses a large number of genes), inducing cell cycle arrest and triggering apoptosis. A number of studies have shown that p53 acts as a repressor of hTERT transcriptional activity (Kusumoto et al., 1999; Kanaya et al., 2000; Xu et al., 2000; Shats et al., 2004) . The mechanism of p53-mediated gene repression involves an interaction with the Sp1 transcriptional activator that prevents it from binding to the hTERT promoter (Kanaya et al., 2000; Xu et al., 2000) . However another study showed that siRNA-mediated depletion of p53 had no effect on hTERT levels in U2OS cells (Lin and Elledge, 2003). Therefore the status of p53 as a bona fide repressor of hTERT remains to be fully clarified. p73 is a member of the p53 family, but plays role in development rather than tumour suppression (Yang et al., 2000). It has been shown to downregulate hTERT transcription, with the β isoform of this protein exerting the most repressive effect on the hTERT promoter (Racek et al., 2005). The GC-boxes in the core promoter region are required for hTERT transcriptional suppression, suggesting that a mitigation of Sp1 transactivation capacity may be involved (Racek et al., 2005; Beitzinger et al., 2006).

BRCA1 is a tumor suppressor protein which functions in a multitude of processes to this effect, including DNA repair, cell cycle checkpoint control and the preservation of genomic stability. BRCA1 also regulates a variety of genes to prevent tumour development (Mullan et al., 2006). A number of studies have demonstrated that BRCA1 can inhibit hTERT transcription (Xiong et al., 2003; Zhou and Liu, 2003) . BRCA1 is a member of a repressive complex that also contains Nmi and c-Myc which compromises the transactivation capability of c-Myc when bound to the E-box on the hTERT promoter (Xiong et al., 2003). It is likely that this particular role of BRCA1 makes an important contribution to its overall tumour suppressor activity.

Wilm's tumour 1 (WT1) protein is another tumour suppressor which bears multiple zinc-fingers and modulates transcription and aspects of RNA metabolism (Yang et al., 2007). WT1 binds to a single consensus sequence situated at -274 in the hTERT promoter and represses hTERT transcription (Oh et al., 1999; Han et al., 2007). Considering that WT1 expression is restricted to genitourinary tissues, the central nervous system, the bone marrow and lymph nodes, it is unlikely to be a universal hTERT regulator (Rivera and Haber, 2005). Myeloid zinc finger-2 (MZF-2) is also a zinc finger TF which is specifically expressed in cells of myeloid origin and is involved in their differentiation (Bavisotto et al., 1991; Murai et al., 1997). There are 4 binding sites for MZF-2 on the hTERT promoter located >500bp upstream of the transcription start site and overexpression of this TF in numerous cell lines downregulates hTERT transcription (Fujimoto et al., 2000).

The E2F family of transcription factors regulates the expression of genes that play a role in cell-cycle progression (DeGregori and Johnson, 2006). E2F proteins interact with DP proteins to generate a TF, the activator/repressor status of which is governed by E2F interaction with one of the pocket (RB) proteins such as pRB (Lees et al., 1993). Overexpression of pRB or E2F1 represses hTERT transcription in squamous carcinoma cell lines (Nguyen and Crowe, 1999; Henderson et al., 2000; Crowe and Nguyen, 2001). E2F-1 binds to the consensus sequence CGCGC located at -174 and -98 on the hTERT promoter (Crowe et al., 2001) . Other members of the E2F and RB families have also been detected binding to the hTERT promoter (Won et al., 2004). This study also revealed the presence of an E2F-RB-HDAC on the hTERT promoter serves as the primary repressive mechanism in cycling somatic cells (Won et al., 2004).

Specifity protein 3 (Sp3) is homologous to Sp1 and utilises the same binding site (Suske, 1999). Unlike Sp1 however, Sp3 has repressive effects on transcription. Sp3 and Sp1 bind to a GC box spanning nucleotides -110 to -102 and negatively regulate the hTERT promoter by recruiting a histone deacetylase complex (Won et al., 2002). For Sp3 to interact with the hTERT promoter and repress transcription, it undergoes deacetylation at a lysine residue (Wooten and Ogretmen, 2005; Wooten-Blanks et al., 2007). One of the important physiological differences between mice and humans is that mice have much longer telomeres in all cells and express telomerase even in somatic cells (Prowse and Greider, 1995; Burger et al., 1997). Cellular senescence in mice does not appear to be caused by telomere attrition (Parrinello et al., 2003). This prompted researchers to determine the cis elements responsible for the much greater repression of hTERT. A comparison of the hTERT and mTERT promoters revealed a nonconserved GC box (CCCCGCCC) in the human promoter (not present in the murine hTERT promoter)

sequence covering nucleotides -7 to -2 which facilitates binding of the Sp1-Sp3 complex to the hTERT promoter (Horikawa et al., 2005). It is speculated that this is partly responsible for the difference in telomerase expression between the 2 species.

Another putative candidate that may contribute to the difference in TERT expression between mice and humans is Activator-protein 1 (AP-1). AP-1 is a heterodimeric transcription factor that is composed of Fos and Jun subunits. It regulates genes in a wide variety of cell processes such as differentiation and proliferation (Shaulian and Karin, 2001). AP-1 has been found to interact with two degenerate binding sites at -1655 and -718 on the hTERT promoter relative to the transcription start site (Takakura et al., 2005). AP-1 expression is ubiquitous in human cells and these promoter sequence elements are not found on the mTERT promoter, suggesting that AP-1 may be responsible for causing hTERT repression in normal human cells.

The transforming growth factor-beta (TGF-β) cytokine has been found to repress hTERT transcription, although the underlying mechanism has not yet been clarified (Katakura et al., 1999; Nozaki et al., 2000; Yang et al., 2001; Liu et al., 2002; Li et al., 2005). SMAD3 is phosphorylated due to an interaction between TGF-β and its cognate receptor. This enables it to gain entry into the nucleus and control gene expression. SMAD3 binds to a GAGA box situated at -262 to -259 on the hTERT promoter and downregulates transcription (Hu et al., 2006; Li et al., 2006). SMAD3 also suppresses the hTERT promoter indirectly by reducing c-Myc expression (Shi and Massagué, 2003). It has also been shown that the SIP-1 TF (which also acts downstream of TGF-β) suppresses hTERT transcription (Lin and Elledge, 2003). TGF-β-activated kinase (TAK1) is a

mitogen-activated protein kinase kinase kinase (MAPKKK) that acts downstream of TGF-β receptor (Yamaguchi et al., 1995). TAK1 causes silencing of the hTERT promoter by stimulating Sp1-mediated recruitment of HDAC2 to the hTERT promoter and also by reducing the quantity of Sp1 bound to the hTERT promoter (Fujiki et al., 2007). Another study has shown that the TGF-β-mediated repression of hTERT is induced by SMAD3 specifically and found no necessity for the E-boxes in TGF-β-conferred repression . One such gene which may be a downstream target of TGF-β is cbfa1 which is a master regulatory transcription factor that modulates the expression of genes associated with osteogenic differentiation (Ducy et al., 1997; Harada et al., 1999). Cbfa1 represses hTERT transcription by interacting with 2 sites (-810 to -787 and -2737 to -2714 in the promoter region (Isenmann et al., 2007).

NFX-1 was first identified as a cysteine-rich transcription factor that binds to a cognate sequence, the X-box, and represses the transcription of the MHC class II genes (Song et al., 1994). It was later discovered it can be alternatively spliced to yield 2 mRNA isoforms called NFX-91 and NFX-123 (Gewin et al., 2004). The NFX-91 splice variant was found to bind to an identified X-box at the hTERT promoter which overlapped with the myc-binding E-box and induces repression. HPV E6-mediated activation of the hTERT promoter was shown to be carried out by ubiquitination and subsequent degradation of NFX1-91 (Gewin et al., 2004; Liu et al., 2005; Katzenellenbogen et al., 2007). NFX1-91 reduces hTERT promoter activity by recruiting the histone deacetylase complex mSin3a (Xu et al., 2008b). Despite the proximity of the E- and X-boxes at the hTERT promoter, it was proposed that NFX1-91 and the Myc-Max activating heterodimer can bind the hTERT promoter simultaneously but the NFX1-91-induced

histone deacetylation inhibits c-Myc transcription (Xu et al., 2008b). Mad1 binds to the Max protein to form a complex that acts in the opposite manner to the c-Myc/max heterodimer in terms of hTERT gene regulation (Ayer et al., 1993). Mad1 binds to the E-boxes in the hTERT promoter to suppress its transcription by deacetylating histones (Gunes et al., 2000; Xu et al., 2001; Xiao et al., 2002; Zou et al., 2006). A switchover occurs from c-Myc-Max to Mad1-Max occupancy at the hTERT promoter during the differentiation of HL60 cells (Xu et al., 2001).

1.4.4. Epigenetic regulation of the hTERT promoter

Methylation of the CpG islands in the vicinity of a promoter region is generally associated with transcriptional silencing of the associated gene (Herman and Baylin, 2003). The hTERT promoter harbours a CpG island and a number of attempts have been made to assess its methylation status by using experimental procedures such as bisulphite sequencing and methylation-sensitive restriction digestion. Some studies indicated that promoter methylation repressed hTERT gene transcription (Lopatina et al., 2003; Shin et al., 2003). However, other findings identified a correlation between hTERT promoter hypermethylation with hTERT gene expression in telomerase-positive cells and seemed to challenge this widely accepted paradigm of gene expression (Guilleret et al., 2002; Nomoto et al., 2002Guilleret and Benhattar, 2003). Other studies yielded variable findings (Devereux et al., 1999; Dessain et al., 2000). To resolve these discordant results, an extensive characterization of the methylation status in the hTERT promoter region was conducted (Zinn et al., 2007). The upstream region of the hTERT promoter in hTERT-positive and hTERT-negative cells was hypermethylated whereas the region surrounding the transcription initiation site was contrastingly hypomethylated in hTERT-postive cells.

Other studies however have demonstrated that methylation in the first 2 exons of the hTERT gene stimulates gene expression. CCCTC-binding factor (CTCF) is an insulator-binding protein that downregulates hTERT transcription (Renaud et al., 2005). Demethylation of 3 particular CpG islands located just downstream of the translation initiation site on the hTERT promoter is required for CTCF interaction (Renaud et al., 2007).

The hTERT locus adopts a repressive chromatin structure and this is the key barrier obstructing its expression in human cells (Wang and Zhu, 2003; Atkinson et al., 2005). A number of histone modifications such as acetylation and H3 phosphorylation have been implicated in hTERT transcriptional activation (Cong et al., 2000; Hou et al., 2002; Ge et al., 2006). Methylation of H3K4 loosens the DNA-histone contacts thereby making it more accessible and acts as a marker to recruit transcription factors to the promoter. SET and MYND domain-containing protein 3 (SMYD3) is a DNA-binding histone methyltranferase and is responsible for H3K4 trimethylation at the 5'end of the hTERT gene (Liu et al., 2007). It was proposed that this modification, serving as a mark for histone acetylation, generates a chromatin environment that facilitates the binding of Sp1 and c-Myc (Liu et al., 2007). A number of histone demethylases that reverse this modification and silence hTERT expression have been identified. Lysine-specific demethylase (LSD1) interacts with the hTERT promoter to demethylate H3K4 and indirectly causes histone deacetylation to induce transcriptional downregulation (Zhu et al., 2008). RBP2 is another histone demethylase that is recruited by Mad1 to the hTERT promoter and it exerts a similar effect (Ge et al., 2009).

1.4.5. Post-transcriptional regulation of hTERT

The hTERT transcript can undergo alternative splicing to produce a number of different transcripts which include the α-transcript (lacking 36 nucleotides from the 5' end) and the β-spliced transcript (which lacks exons 7 and 8) (Kilian et al., 1997; Ulaner et al., 1998). These negatively regulate telomerase activity which is only attributable to the full-length transcript (Colgin et al., 2000). A number of studies have shown the alternative splicing of hTERT is a biologically significant event. The hTERT promoter possesses a number of hypoxia-responsive elements, and it has recently been shown that hypoxia-inducible factor-1-α (HIF-1α) associates with these and with the downstream elongating transcriptional complex (Anderson et al., 2006). Furthermore, the HIF-1α interaction was shown to influence alternative hTERT transcript splicing to favour the processing of the full-length hTERT transcript. The frequency of alternative splicing of non-functional isoforms is increased during the chemically-induced differentiation of HL60 cells, adenocarcinoma cells and fetal human colon cells (Fajkus et al., 2003; Liu et al., 2004a). hTERT is differentially spliced in a tissue-specific manner during human development (Ulaner et al., 1998).

1.4.6. Post-translational modification of hTERT

A number of kinases have been identified which regulate telomerase activity by phosphorylating the hTERT protein. The Akt (protein kinase B family) is comprised of 3 serine/threonine specific protein kinases that are implicated in processes such as growth regulation, insulin signaling and brain development (Gonzalez and McGraw, 2009). Akt1 in particular is associated with growth stimulation and numerous studies have found that

it activates telomerase activity through the phosphorylation of hTERT (Kang et al., 1999; Breitschopf et al., 2001; Kimura et al., 2004). In addition to its activation of hTERT transcription, oestrogen also upregulates telomerase activity by phosphoryating the hTERT protein through the Akt-signalling pathway in endothelial progenitor cells and PC12 cells (Du et al., 2004; Imanishi et al., 2005). The c-Abl tyrosine kinase regulates proteins that participate in processes such as cell division, adhesion and differentiation (Bradley and Koleske, 2009). It has been demonstrated that c-Abl uses its SH3 domain to interact with a proline-rich segment of the hTERT polypeptide and the resulting phosphorylation leads to a reduction in telomerase activity (Kharbanda et al., 2000). A feature of chronic myelogenous leukemia is the presence of a Philadelphia chromosome which results in a Bcr-Abl fusion protein with constitutive kinase activity (Goldman, 2004). Downregulation of the BCR-Abl fusion product results in increased telomerase activity (Bakalova et al., 2004).

The protein kinase C (PKC) family consists of over 10 isoforms and is involved in regulating a wide variety of physiological activities. PKC-ζ and -α activate telomerase through hTERT phosphorylation in nasopharyngeal and breast cancer cells (Ku et al., 1997; Li et al., 1998; Yu et al., 2001). The precise mechanism of how hTERT phosphorylation modulates telomerase activity has not yet been fully elucidated but one study has provided evidence that PKC-mediated phosphorylation of hTERT is required for its interaction with hsp90 (Chang et al., 2006). The addition of ubiquitin to a protein serves to mark it for degradation by a proteasome. hTERT is ubiquitinated by MKRN1 and its overexpression provides another means of mitigating telomerase activity (Kim et al., 2005).

1.4.7. Regulation of the assembled telomerase complex

Most telomerase regulation occurs at the level of hTERT transcriptional regulation. Another mechanism by which telomerase is regulated in humans is through modulation of the accessibility of the assembled functional telomerase complex to its telomere substrate. Telomeres have been postulated in yeast to have extendible and non-extendible length-dependent states in terms of telomerase activity (Teixeira et al., 2004). There are a number of structural features specific to telomeres which serve as a means of regulating extension by telomerase. The prevalence of guanine residues in telomeres results in the formation of DNA quadruplexes or quartets by the ssDNA overhangs in vitro (Parkinson et al., 2002). These quadruplexes are helical structures which are stabilized by a tetrad of guanines from consecutive TTAGGG repeats bonded to each other by Hoogsten hydrogen bonding. The plane of the tetrad is usually centered on a divalent metal cation which coordinates the quadruplex strucuture (Williamson et al., 1989). There is evidence that these structures are formed in vivo (Schaffitzel et al., 2001; Chang et al., 2004; Paeschke et al., 2005). Thus G-quadruplexes could potentially represent a barrier to telomerase extension in addition to the shelterin components.

As mentioned previously, POT1 specifically binds to the ssDNA overhang and prevents telomerase activity by sequestering this part of the DNA and preventing it from being utilized as a substrate by telomerase (Loayza and De Lange, 2003). POT1 interaction with telomeres is at least partly facilitated by the presence of bound TRF1 on the dsDNA component of the telomeres (Smogorzewska and de Lange, 2004). Longer telomeres in human cells, therefore, lead to greater amounts of bound TRF1 which increases the

probability that POT1 will bind to the telomeres. Currently proposed models suggest 2 ways in which POT1 negatively regulates telomerase activity. In the first model, POT1 preferentially binds to its cognate sequence at the 3'end and this may prevent its use as a substrate by the telomerase complex (Baumann and Cech, 2001; Lei et al., 2002). An alternative model has been proposed in which POT1 interacts with ssDNA of the displaced d-loop that is a consequence of T-loop formation (Loayza et al., 2004). POT1 could therefore stabilize the d-loop and through these means prevent exposure of the 3'end that is required by telomerase. However, another study implicated POT1 in the positive regulation of telomere elongation (Colgin et al., 2003). Particular events such as a specific stage of the cell cycle may be responsible for determining the effect of POT1 on telomerase accessibility.

In yeast, a 'protein-counting' model has been postulated as a means of coupling telomere accessibility to telomere length involving the telomere binding protein Rap1. A similar principle applies to human telomeres. TRF1 and TRF2 negatively regulate telomerase activity in cis (van Steensel et al., 1997; Smogorzewska et al., 2000). The amount of TRF1 bound to the telomere is proportional to the telomere length (Loayza et al., 2003). As telomere length is reduced, this leads to commensurately lesser amounts of bound TRF1, which in turn reduces the possibility of obstructing telomerase access to the telomere. The HPV E6 protein can increase telomerase activity by a mechanism that depends on its interaction with telomeric DNA (Liu et al., 2009). Although the function of TERRA RNA has yet to be fully elucidated it has been considered that it negatively regulates telomerase activity in view of the fact TERRA levels are lower in a number of telomerase positive tumours (Schoeftner et al., 2008). Furthermore it has been found in

yeast that TERRA upregulation due to impaired function of Rat1-1 exonuclease resulted in shorter telomeres (Luke et al., 2008). It is therefore possible that it interacts with the complementary sequence of the hTR non-coding RNA and prevents it from using the G-strand as a substrate to extend the telomere.

1.4.8. Aim of Project

In summary, telomerase is extensively regulated throughout the various stages of its assembly. Given its widespread presence in human cancers, the identification of novel telomerase regulators could be of potential therapeutic significance. It is also of interest to determine the factors that induce a cell to activate telomerase or the ALT recombination-based TMMs to overcome crisis. In this project, efforts will be made to fulfill these objectives via the use of stable isotope labeling with/of amino acids in cell culture (SILAC). SILAC is a relatively novel proteomics cell culturing technique that enables one to quantitatively compare the proteomes of 2 cell populations (SILAC; see section 2.2.1 for more details). In this project, 2 genetically similar cell lines that are telomerase-positive and telomerase-negative have their proteomes contrasted using this comparative proteomics approach with the intent of isolating proteins that activate/repress either of these TMMs. The identification of such a regulator will be subsequently validated by further molecular characterization of its function in relation to telomerase regulation.

Chapter 2

Experimental Procedures

2.1. Initial Characterisation of the 6G and 1-3C cell lines.

2.1.1. Cell culture conditions

The JFCF cell line was originally generated by isolating Jejunal Fibroblasts from a Cystic Fibrosis patient and then immortalizing these cells with the pRSV-T plasmid that expresses the large T antigen of the SV40 virus. The 1-3C and 6G cell lines are two independent clones that emerged from the pre-immortalised population of transformed JFCF cells and were kindly provided by Roger Reddel of the Children's Medical Research Institute, Sydney, Australia. Cells were cultured in Dulbecco's modified eagle's medium (D-MEM; Gibco, USA) supplemented with 10% dialysed fetal bovine serum (Gibco, USA) and 0.1% gentamicin (Gibco, USA). Both cell lines were cultured at 37°C and 5% CO_2. All other cell lines were cultured in the same fashion, except for those specially labelled for SILAC analysis. For cell culture conditions pertaining to the SILAC experiments, see section 2.2.2.

2.1.2. Detection of integrated SV40 sequences in the genomes of the immortalised JFCF cells

Detection of SV40 sequences in the JFCF clones was performed via a standard Southern blotting procedure. Briefly, 40µg genomic DNA of each sample was digested overnight at 37°C with 80units of *Xba1* (Roche, USA) and 8µg digested DNA was loaded onto a 0.8% agarose gel in 1×Tris-acetate EDTA (TAE) buffer. DNA size markers were

comprised of λ *HindIII*-digested and ΦX174 *HaeIII*-digested DNA (New England Biolabs, USA). The DNA was then electrophoresed at 30V for 16 hours and the gel was subsequently denatured and neutralised. It was subsequently transferred onto Hybond N+ (Amersham Pharmacia, UK) membrane by capillary blot with 20x saline-sodium citrate (SSC) buffer overnight and DNA was UV fixed by auto-crosslinking in a Stratalinker (Stratagene, USA). Prehybridisation was carried out with 20mls of 5×SSC, 10× Denhardt's solution, 0.5% SDS, 50μg/ml sonicated herring sperm DNA at 65°C for 1hour. Hybridisation was then performed overnight at 65°C in 20mls hybridisation solution (50% formamide, 5× SSC, 0.5% SDS, 50μg/ml sonicated herring sperm DNA) containing 2x 10^6 counts/ml of ^{32}P-dCTP labelled pSV2 plasmid, which harbours SV40 sequences. The filter was washed three times in 2×SSC/0.1% SDS at 65°C and autoradiographed on Kodak Biomax MR or MS X-ray film for 4-72h at -80°C.

2.1.3. Telomere repeat amplification protocol (TRAP) assay

Cell lysates were obtained using the 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) detergent lysis method, and 2 μg of total protein was used in each assay. The protein concentration of lysates was measured using the Bio-Rad Protein Assay kit (Hercules, USA). The PCR-based TRAP assay for telomerase activity was performed as previously described (Piatyszek et al., 1995). Amplification products were separated on a 10% non-denaturing polyacrylamide gel, stained with SYBR green I (Molecular Probes, USA.) and visualized using a Storm 860 imager (Molecular Dynamics, USA.).

2.1.4. Terminal restriction fragment (TRF) analysis

Genomic DNA was prepared from each cell line, and 40 μg was digested with the restriction enzymes *Hin*fI and *Rsa*I (New England Biolabs, USA). The recognition sequences of both enzymes are not located within telomeric DNA. Quantified samples (1μg) were then loaded onto a 1% agarose gel in 0.5 × Tris-borate-EDTA (TBE) buffer and separated using a CHEF-DR II pulsed-field electrophoresis apparatus (Bio-Rad, Germany). 0.5 X TBE buffer was circulated at constant temperature of 14°C with a ramped pulse speed of 1 to 6 s at 200 V for 14 h. The gels were dried, denatured, hybridized to a [γ-^{32}P] ATP end-labelled telomeric DNA oligonucleotide probe, $(TTAGGG)_3$, and exposed to XAR film (Kodak, Japan) at -80°C for 18 h.

2.2 Techniques used for the SILAC analysis

2.2.1. Explanation of the SILAC procedure

Quantitative proteomics approaches can essentially be classified on the basis of whether or not the peptides are labelled prior to MS analysis. The label-free approach is less frequently employed and has a number of technical disadvantages in contrast to the labeling approach. Two examples of label-free quantitative proteomics that have been developed include protein-correlation profiling and quantitation by means of comparison to a 'pseudo-internal standard' (Andersen et al., 2003; Tabata et al., 2007). Labelling procedures can be sub-divided into *in vitro* and *in vivo* techniques. *In vitro* labeling preceded *in vivo* labelling and involves the modification of extracted protein with an isotopic tag. The most commonly used variant of this procedure involves the use of isotope-coded affinity tags in which cysteine residues are tagged with a moiety that contains either eight or zero deuterium atoms and a biotin group that can be used to

isolate peptides containing this tag for further analysis (Gygi et al., 1999). In this project, an in vivo labeling strategy called the stable isotope labeling of amino acids in/of cell culture (SILAC) will be employed (Ong et al., 2002).

In SILAC the 2 cell populations one wishes to compare are grown in media that are identical with the exception that one contains at least one isotopically labeled variant of an essential amino acid (Figure 2.1). After ~6 population doublings, the labelled amino acid is 98-99% incorporated into the cellular proteome. A number of modified amino acids have been exploited in the SILAC methodology such as leucine, arginine, lysine and tyrosine (Ong et al., 2003). The proteins from both cell samples can then be combined in equal amounts, resolved by SDS-PAGE and proteins of similar mass in a specific band can be subjected to tryptic digestion and MS analysis. Trypsin cleaves the C-terminal bond of lysine and arginine in a polypeptide and thereby ensures that all the resulting peptides have an arginine or a lysine. In a particular MS run, a peak doublet will consist of a peak signal for the unlabelled peptide and one for the isotopically labelled peptide. Spectral sampling is used to determine the relative abundance of a given peptide in the sample (Liu et al., 2004b). The ratio of peak intensities of both respective peptides can then be determined. This is used, in conjunction with other peak intensity ratios of peptides for a given protein, to calculate how the level of a protein varies in one population of cells with respect to another cell population.

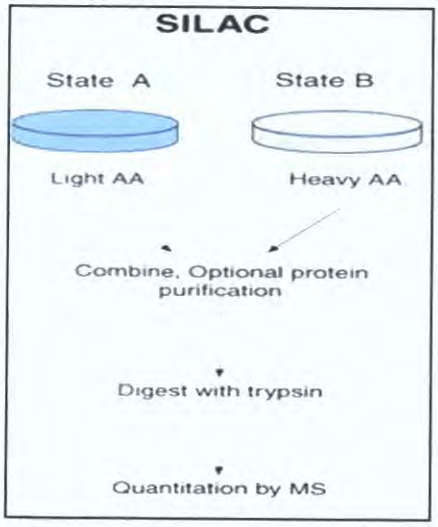

Figure 2.1. Outline of the SILAC procedure.
The SILAC proteomic technique relies upon the incorporation of a heavy isotope labelled amino acid (in this particular study is $^{13}C_6$ L-Lysine HCl used) into all of the proteins in the 'heavy' AA cell line (State B is telomerase-positive in our experiments). The telomerase-negative fibroblasts (State A) are grown in normal medium ('light' AA cell line). Extracts from both cultures are then combined and analysed by SDS-PAGE, followed by in gel tryptic digestion and mass spectrometry.

2.2.2. SILAC cell culture conditions

SILAC was performed using SILAC Protein identification and quantitation kits (Invitrogen,, USA) according to the manufacturer's instructions. Briefly, to prepare SILAC medium containing heavy arginine, 100mg [$^{13}C_6$, $^{15}N_4$]-L-arginine and 100mg of L-lysine were each added to minimal D-MEM medium which lacks these two amino acids, supplemented with 10% dialysed fetal calf serum, L-glutamine and penicillin-streptomycin. The medium was then filtered with a 0.22μm filtration apparatus (Corning, USA). This protocol was also adopted for the generation of SILAC medium containing heavy lysine ($^{13}C_6$-L-lysine) and regular light L-arginine and for the generation of medium containing light amino acids only. Cells were transferred from regular D-MEM

to the SILAC media by using the following approach. Semi-confluent cells grown in D-MEM medium in T75 flasks (Nunc, USA) were trypsinized. Regular D-MEM medium was then added and the cells were centrifuged at 100×g for 5 minutes. The supernatant was subsequently removed and the cells were then re-suspended in the appropriate SILAC medium. Cells were then grown again for 6 population doublings prior to carrying out the initial analyses to assess the extent of incorporation of the heavy amino acids.

2.2.3. Pairing-on membrane (POM) assay

The POM assay was carried out as previously described with minor modifications (Akhmedov et al., 1995). A 7×10cm nitrocellulose membrane (Sigma, USA) was wet on one side with 2×SSC buffer containing ϕX174 virion ssDNA at a concentration of 100μg/ml (New England Biolabs, USA). The membrane was then allowed to dry out to ensure adhesion of ssDNA to the membrane. 50μg protein for each cell line was separated with Novex NuPage 4-12% Bis-Tris Midi gel (Invitrogen, USA) using 1×MOPS buffer (Invitrogen, USA) and a Dual Mini Slab apparatus (Atto, Japan). These proteins were then transferred by western blot to the ssDNA-coated nitrocellulose membrane using the Mini-Protean-3 gel tank system (Bio-Rad, Germany). The membrane was subsequently incubated with a pre-incubation buffer (33mM Tris.HCl, 2mM dithiothreitol, 1mM $MgCl_2$ and 5% (w/v) BSA) at room temperature for 30 minutes. The membrane was then given 3×1minute washes with incubation buffer (33mM Tris.HCl, 2mM dithiothreitol, 10mM $MgCl_2$). The membrane was then incubated with hybridization buffer (2.5μg biotinylated ϕX174 RFII dsDNA, 0.6μm dNTPs, 0.1mM ATP-gamma-S, 33mM Tris.HCl, 2mM DTT and 10mM $MgCl_2$) for 2 hours. The

membrane was given 2×5 minute washes in wash buffer (0.5% SDS, 5mM EDTA and 10mM Tris). The membrane was then incubated with proteinase K buffer (0.1mg/ml proteinase K, 0.5% (w/v) SDS, 5mM EDTA and 10mM Tris) at 37°C for 1 hour. The membrane was then given 4×10 minute washes with wash buffer II (2×SSC, 0.1% SDS) at 65°C. To assess whether or not biotinylated ϕX174 DNA had been transferred to the membrane by means of strand exchange, the North2South chemiluminescent substrate hybridization kit (Pierce Biotechnology, USA) was used and the manufacturer's guidelines were followed.

2.2.4. Protein extraction and 1-D SDS PAGE for SILAC

Nuclear proteins were lysed from the 6G and 1-3C cell lines that had been cultured in their respective different SILAC media as previously described. Nuclear extracts were harvested using the NE-PER kit (Pierce Biotechnology, USA) according to the manufacturer's instructions. A discontinuous gradient gel was used to separate proteins before being subjected to mass spectrometry. A gradient polyacrylamide gel provides two advantages in this regard. A gradient gel allows for superior resolution of high Mr proteins and those of very similar Mr in contrast to fixed acrylamide percentage gels. To construct the discontinuous gradient gel, 6 different polyacrylamide percentages were used (including that of the stacking gel section). Their composition is described in table 2.1. 500μg of nuclear extract harvested from each cell line were combined and loaded onto a single well of a discontinuous gradient gel in Bio-Rad Protean II xi electrophoresis chamber (Bio-Rad, Munich, Germany). The samples were then subjected to electrophoresis at 200V for 4 hours. Gels were then stained using Coomassie Blue G-250.

Table 2.1 Composition of sections of SDS-PAGE gel used to resolve proteins prior to MS analysis.

Gel %	4%	6%	8%	10%	12%	14%
Bis	0.1%	1.5	1.95	2.5	2.9	3.4
acrylamide	4%	6%	8%	10%	12%	14%
0.5M Tris,pH 8.8	0.125M	-	-	-	-	-
1.5M Tris, pH 6.8	-	0.375M	0.375M	0.375M	0.375M	0.375M
SDS	1%	1%	1%	1%	1%	1%

2.2.5. Coomassie Blue G-250 staining

The polyacrylamide gels used for SILAC experiments were stained with Coomassie Blue G-250. The gel was placed in 50-100 ml fixation solvent (50% (v/v) methanol, 2% (v/v) phosphoric acid) for approximately 1-2 hours with rocking. After this step the gel was then washed three times with distilled water for ten minutes each. The gel was then treated with incubation solvent (34% (v/v) methanol, 2% (v/v) phosphoric acid and ammonium sulphate 17% (w/v)) for one hour with agitation. Coomassie Blue G-250 was then added at a final concentration of 0.025% and the gel was left to rock overnight. The gel was then washed three times for ten minutes each in distilled water, twice with 25% methanol for 5 minutes and three times in distilled water once more again. All gels were then sealed and stored at 4°C until further analysis.

2.2.6. In-gel tryptic digestion

The gel lane in which the combined, differently labelled nuclear lysates were resolved was divided equally into sections. These excised gel pieces were then cut into small pieces and then placed into a 0.5ml Eppendorf tubes containing 100µl of 200mM NH_4HCO_3, pH 7.8. The NH_4HCO_3 was then removed with a pipette, taking care not to absorb any of the gel pieces. The 200mM NH_4HCO_3 solution was replaced and this step was repeated until visual evidence of Coomassie G-250 staining was no longer present. These tubes were then agitated in a thermomixer at 37 °C for 15 minutes. The tubes were then centrifuged at 6000g for 10 seconds and the NH_4HCO_3 was removed. 100µl of a solution consisting of 200mM NH_4HCO_3 (pH 7.8)/Acetonitrile in a 4:6 ratio was then added to each tube. The tubes were then agitated at 1000rpm in a thermomixer at 37°C for 30 minutes. The solution was then removed from each tube by pipette and was replaced with 100µl of 50mM NH_4HCO_3. The tubes were then agitated at 1000rpm in a thermomixer at 37°C for 30 minutes. The samples were then centrifuged at 6000g for 10s and the solution was removed by pipette. 100µl acetonitrile was then added to each tube and left for 5 minutes on the bench. Tubes were then centrifuged at 6000g for 10 seconds and the supernatant was removed by pipette.

The samples were then subjected to successive reducing and alkylation steps. For the reducing step, 100µl of 10mM DTT in 100mM NH_4HCO_3 was added to each sample and they were then incubated at 56°C for 1 hour. The samples were then centrifuged at 6000g for 10s and the supernatant was removed by pipette. For the subsequent alkylation step,

100µl of a 100mM NH_4HCO_3 containing 50mM iodoacetamide in was added to each sample. The samples were then incubated on a rack and covered in tin-foil. They were incubated in the dark for 30 minutes to optimise alkylation. Samples were then centrifuged at 6000g for 10s. The samples were washed by adding 200µl of 100mM NH_4HCO_3 and were agitated in a thermomixer at 1000rpm for 15 minutes. The tubes were then centrifuged at 6000×g for 10s and the supernatant was removed. The samples were then washed again with a solution consisting of both 50mM NH_4HCO_3 and acetonitrile in 1:1 ratio. Samples were centrifuged again at 6000g for 10s and the supernatant was removed. 100ul of acetonitrile was then added to the samples and these were then agitated at 1000rpm for 5 minutes in a thermomixer.

For the protein digest, an active protease solution was prepared. A stock solution of trypsin was prepared by adding 100µl of trypsin reconstitution buffer (Promega, USA) to 20ug trypsin (Promega, USA). To 10µl of this new tryspin solution, 500µl of 50mM NH_4HCO_3 was added. 50ul of this solution was then added to each sample and was placed on a thermomixer and left to mix at 37 °C overnight. After the overnight incubation, 10µl of 5% formic acid was added to each sample and they were incubated at room temperature for 20 minutes. The samples were then centrifuged at 6000g for 10s and the eluants containing the digested peptides were transferred to new tubes. 20µl of a solution consisting of 2% formic acid and 60% acetonitrile was added to the remaining gel pieces and the samples were left at room temperature for 5 minutes. The samples were then mixed thoroughly and centrifuged at 6000g for 10s. The supernatant was then transferred to the tubes containing the first eluant. The samples were then reduced to a much smaller volume of 2-5µl by centrifuging in a speedivac apparatus. 10µl of 1%

formic acid was then added to each of the tubes and this was then vortexed and centrifuged. The digested peptides were then transferred to MS vials for MS analysis.

2.2.7. MS analysis to identify proteins detected in POM assay

The peptide mixtures were analysed by LC-MS/MS. 3µl of the peptide sample was injected into a CapLC nanoHPLC system (Waters, UK) which was connected to a quadropole time-of-flight (Q-TOF) Ultima API mass spectrometer (Waters, UK). The qTOF MS instrument format is typically more suited to sensitive protein quantitation than the quadropole ion-trap format due to the space-charge restrictions of the latter (Ong et al., 2003). Mascot software (version 2.1.3; Matrix Science, USA) was employed to search the Ensembl database (Homo sapiens) for proteins that match the peptide spectra acquired during the course of the qTOF mass spectrometry run. The original files (.raw format) generated by the qTOF mass spectrometry run had to be modified before they were used as inputs in the mascot protein search algorithm to create results in .html format that would be compatible with the MSquant software. They were converted to peak list (.pkl) files to enable the MSquant program to discern the MS/MS and MS spectra. This was achieved by using this file as input to be altered by a perl script which is freely available for downloading in the Q-TOF entry on the MSquant homepage at www.sourceforge.net.

For the search settings, a tolerance threshold of 50ppm was set for the peptide precursor ion and 0.5Da for the fragment ion tolerance. The probability of error threshold for the identification of a particular peptide was set at $p<0.02$. This is the mascot identity threshold which can be mathematically defined as $-10\log_{10}[20x(p/n)]$. In this equation p is equal to the probability of a random peptide match and n is the number of total peptide

candidates. The variable modifications also specified were carbamidomethylation of cysteine and oxidation of methionine. The .html output file was then saved as this would be later used as an input in the MSquant software procedure. Mass spectral data were processed into peak lists containing the m/z value of each precursor ion and the corresponding fragment m/z value and intensity. In order to perform the relative protein quantification the MSquant software package was employed (Peter Mortensen, University of Southern Dennmark; available for download at www.sourceforge.net). The $^{13}C_6$-Lys modification was included in the list of parameters. Both the .raw and .html files (generated by the Mascot search algorithm) were used as inputs.

2.2.8. Mass spectrometry for global nuclear proteomics analysis

Protein bands were excised and subjected to in-gel tryptic digestion as previously described above with modified porcine trypsin (Promega, MA, USA). 1µl of the eluted peptides was then analysed by means of LC-MS/MS. Chromatographic separation of peptides was achieved by means of a 1hr acetonitrile gradient on an Ultimate 3000 HPLC system (Dionex, UK). This was linked to a HCT Ultra ion-trap mass spectrometer (Bruker Daltonics, UK) that used electrospray ionization to induce peptide fragmentation. The MS and MS/MS spectra were then generated by processing the data with the Bruker Data analysis software programme. To confine data acquisition to highly differentially expressed proteins that are more likely to be of interest, the 'stable isotope labelling experiment' (SILE) parameter was applied during the data acquisition phase. SILE is used for the selective acquisition of data below/above or between 2 specifically set intensity ratio values. The corresponding .mgf files were then searched using the X!Tandem search algorithm (available at www.thgpm.org) against the human SWISS-

PROT protein database. The following parameters were applied in searching for proteins: Fragment mass error: 0.5 Da, parent mass error: +250ppm to -250ppm. The following refinement modifications were employed to perform the search: Round 1: oxidation (M), oxidation (W), deamidation (N), deamidation (Q). Round 2: dioxidation (M), dioxidation (W). The residue modifications used as input parameters can be found in appendix 3. Proteins identified via this search were checked for peptides to determine if corresponding E-values did not exceed -3. MS spectra were then examined to identify the corresponding peak doublets and determine the (heavy/light) peptide ratio.

2.3. Validation of SILAC results and further investigations

2.3.1. RNA extraction and determination of concentration

RNA was extracted from the 6G, SUSM-1 and 1-3C cell lines by using the RNeasy kit (Qiagen, Germany). 3×10^6 cells were used in extracting RNA from each cell line and the manufacturer's guidelines were then followed to conduct the remainder of the protocol. Fractions of the RNA samples were diluted at a ratio of 1:100 and the respective absorbances were measured at 260nm and 280nm wavelengths using a Biophotometer spectrophotometer (Eppendorf, Germany). The absorbance measured using a 260nm wavelength (A_{260}) was used to assess the concentration. The A_{260}/A_{280} ratio is a measure of RNA purity and these average values (of 3 readings) for the 3 cell lines ranged from 1.68 to 2.04.

2.3.2. Reverse transcription to generate cDNA for real-time PCR reactions

To generate cDNA from the extracted RNA, the RNA sample was first treated with DNase to remove any contaminating DNA fragments. DNase reactions each consisted of 4μg RNA, 10×DNAase reaction buffer, and 2μl RQ1 RNase-Free DNAase (Promega, USA) at a final concentration of 1 unit/μl. The total reaction volume was made up to 20μl with nuclease-free water (Sigma, Germany). Each reaction was first incubated at 37 °C for 30 minutes and was then terminated by adding 2μl stop solution (Promega, USA). The reaction was then heated at 65 °C for 10 minutes. For the reverse transcription step, the following was then added to the DNAse-treated sample: 1μg $dT_{(12\text{-}18)}$ oligonucleotides (Invitrogen, USA) and 20 μmol dNTPs. The final reaction volume was 26μl and this was then heated at 65 °C for 5 minutes. It was centrifuged briefly and then cooled on ice. The following was then added to the sample: 5×First strand buffer, dithiothreitol (for a final concentration of 10μM) and 80 units of Recombinant RNaseOUT RNasin Ribonuclease inhibitor (Invitrogen, USA). The final reaction volume was 40μl which was mixed thoroughly and then incubated at 37 °C for 2 minutes. 200 units of M-MLV RT (Invitrogen, USA) were than added to the sample. The sample was then heated at 37 °C for 50 minutes followed by 70 °C for 15 minutes.

2.3.3. Quantitative Real-time PCR

The primers used for the genes examined in the real-time PCR in this project are listed in table 2.2. Primer specificity was verified by agarose gel electrophoresis. To perform Quantitative real-time PCR, the QuantiTect Sybr Green Kit (Qiagen, Germany) was used. As per the manufacturer's instructions, each assembled reaction consisted of: 2X QuantiTect SYBR Green mastermix, 0.5mM each of forward and reverse primers and

500ng cDNA. Nuclease-free water was then used to make up the final reaction volume total to 20µl. Reactions were prepared in 20µl volume Lightcycler capillaries (Roche, USA). The real-time PCR procedure was initiated with a preincubation step at 15°C for 15 minutes. This was then followed by an amplification step consisting of: 94°C for 15s, 64°C for 20s and 72°C for 20s. Melting curve analysis was carried out after 35 cycles using Lightcycler software, version 2.14 (Roche, USA). Quantitative real-time PCR was carried out for the β-actin gene initially and this represented the reference sample. Relative quantification was accomplished by normalising the values for all three genes to that of the reference sample.

Table 2.2. Primers used for quantitative reverse transcriptase PCR.

Gene	Primer orientation	Melting temperature	Sequence
RuvBl2	Forward	63.6	5' GCAAACTGACCCTCAAGACC 3'
	Reverse	63.6	5' GAGCCCATAGCGTCGTAGTC 3'
Stat1	Forward	63.8	5' TCGGGGAATATTCAGAGCAC 3'
	Reverse	64.2	5' CACTCTTTGCCACACCATTG 3'
Ube2N	Forward	63.6	5'CCAGGCCTTGTTAAGTGCTC 3'
	Reverse	63.7	5'GGAAGTCTTGGCAGAACAGG 3'

2.3.4. siRNA transfection

siRNA transfection was carried out using the siPORT *Lipid* Transfection Agent cells were seeded out in 12-well plates at a density of 2×10^5 cells per well and were then incubated in medium overnight. siRNA transfection was performed 24 hours later when cells were approximately 30%-70% confluent. For each well, 4µl siPORT *Lipid* transfection Agent was diluted in Opti-MEM I up to a total volume of 15µl. This (Solution 1) was then incubated at room temperature for 20 minutes. Similarly, 10µl of 1µM *Silencer* Select RuvBl2 siRNA (siRNA ID=s21309; Ambion, USA) was made up to 185µl with Opti-MEM I and this (solution 2) was then incubated at room temperature for 15 minutes. After concluding their incubation phases, both solutions (1) and (2) were combined and incubated at room temperature for 20 minutes to form transfection complexes. Cells were washed with Opti-MEM prior to transfection and then 800µl of Opti-MEM was added to each well. The 200µl transfection mixture was then added to each well. Cells were then maintained under standard cell culture conditions for 4 hours. The final siRNA concentration was therefore 10nM. After this period, the Opti-MEM was replaced with regular D-MEM medium. Cells were harvested the following day at 100% confluency to extract protein using the M-PER kit (Pierce Biotechnology, USA). For Ets2 siRNA transfection, the Ets2 validated Stealth RNAi Duplex#1 (product no. = 461590; Invitrogen, USA) was used. The Ets2 transfection was carried out by using the same procedure outlined for RuvBl2, except the final siRNA concentration in each well was 100nM.

2.3.5. Protein electrophoresis and Western blotting

Total-cell lysates from transfected cells were harvested with the M-PER protein extraction kit (Pierce Biotechnology, USA). Protein concentration was determined using the BSA concentration assay. A BCA kit (Pierce Biotechnology, USA) was used to accomplish this following the instructions as specified by the manufacturer. To determine concentration samples were added to a 96-well microplate and absorbance at 562nm wavelength was measured by a Synergy HT Multi-mode microplate reader (Biotek, USA) using KC4 software, version 3.4 (Biotek, USA).

Two different polyacrylamide gels were prepared for protein electrophoresis prior to Western blot transfer. A 12% polyacrylamide gel was prepared for RuvBl2 and Ets2 western blot detection. An 8% polyacrylamide gel was used to detect the hTERT protein, owing to its greater mass (127kD). The part of the gel comprising the loading wells was prepared with a 4% stacking gel. The compositions of all three polyacrylamide gels are outlined in table 2.3. Loading samples were prepared by combining 6×SDS loading buffer, 150μg protein and were made up to a total volume of 20μl with dH_2O. Before loading, they were heated at 95°C for 4 minutes. Gels were then run at 120V for 4 hours in a Dual Mini Slab apparatus (Atto, Japan) in 1×SDS running buffer (25mM Tris-HCl, 200mM glycine and 0.1% SDS). A large amount (150μg) of protein was used as hTERT is difficult to detect due to its very low abundance.

Table 2.3. Reagents used to generate SDS-polyacrylamide gels for protein electrophoresis.

Abbreviations: APS-ammonium persulphate, SDS-sodium dodecyl sulphate, TEMED-Tetramethyethylenediamine.

	4% stacking gel	**8%**	**12%**
Tris, pH 8.8	-	0.375M	0.375M
Tris, pH 6.8	0.125M	-	-
Bis-acrylamide	4% (v/v)	8%(v/v)	12% (v/v)
APS	1% (v/v)	1% (v/v)	1% (v/v)
TEMED	0.04%(v/v)	0.04%(v/v)	0.04%(v/v)
SDS	1% (v/v)	1% (v/v)	1% (v/v)

2.3.6. Western blotting

After resolving proteins on an SDS-PAGE gel, they were then transferred to a nitrocellulose membrane (Bio-Rad, Germany). The gel was sandwiched between two pieces of filter paper (Whatman, USA) on either side. Western transfer was then carried out using a Mini-Protean-3 gel tank system (Bio-Rad, Germany) and was performed at constant 250mA for 1 hour in Western transfer buffer (25mM Tris, 192mM glycine, 10% (v/v) methanol). The membrane was the stained with Poinceau-S (Sigma, Germany) and was visually inspected to determine if transfer had occurred. Membranes were then gently agitated for 40 minutes in a 5% BSA, 1×Tris-buffered saline-Tween (TBST) blocking solution (5% non-fat dry milk, 20mM Tris.HCl (pH 7.5), 150mM NaCl and

0.05% (v/v) Tween-20), with the blocking solution being replaced every 10 minutes. The membrane was then incubated overnight at 4°C with the relevant primary antibody in 1×TBST blocking solution. The antibody concentrations used were as follows: hTERT antibody (Product no. 5181; Abcam, UK)-1:500, RuvBl2 antibody (Product code: ab36569; Abcam, UK)-1:500, Ets2 antibody (sc-351; Santa Cruz Biotechnology, USA)-1:200 and β-actin antibody (Sigma, Germany)-1:7500. The membrane was then given 4×10 minute washes with 1×Tris BST (20mM Tris.HCl, (pH 7.5), 150mM NaCl and 0.05% (v/v) Tween-20) solution and was then incubated with an appropriate horseradish peroxidase-linked secondary antibody (for 1 hour at room temperature). Rabbit secondary antibody diluted 1:2000 in blocking solution was used for Ets2 and RuvBl2. Mouse secondary antibody was used for hTERT (dilution factor-1:2000) and β-actin (1:7500). The membrane was then given another series of 4×10 minute washes with 1×TBST solution. Blots were developed using Supersignal West Pico Chemiluminescent Substrate kit as per the manufacturer's guidelines.

2.3.7. ChIP protocol

6G cells were serum-starved for 24 hours when their confluence was estimated to be 30-70%. Epidermal growth factor/or vehicle alone was added to the culture medium to give a final concentration of 5ng/ml. To induce DNA-protein complex cross-linking, cells were fixed with 1% formaldehyde for 10 minutes at 37°C. The unreacted formaldehyde was quenched and cross-linking was inhibited by adding glycine at a final concentration of 0.125M to each flask. Cells were then washed twice with ice-cold 1×phospho-buffered saline (PBS) with protease inhibitors added. Cells were then detached from the flask by scraping and were transferred (in ice-cold 1×PBS, with protease inhibitors added) to a

15ml sterile tube (BD Biosciences, USA) tube on ice. These were then centrifuged at 290×g for 5 minutes at 4°C and the supernatant was removed and discarded.

Cells were then resuspended in 400µl SDS lysis buffer (1% SDS, 10mM EDTA and 50mM Tris Hcl, pH 8.1; protease inhibitors were added prior to use) and incubated on ice for 10 minutes. Each sample was given 8×10-second sonications with a 1 minute interval between each sonication. The sample was then transferred to a 1.5ml eppendorf tube (Eppendorf, Germany) and centrifuged at 17,969×g at 4°C for 10 minutes. The supernatant was retained and was made up to 2 ml with ChIP dilution buffer, with 40 µl being removed as inputs. At this point the concentration of DNA was obtained by spectrophotometry. Appropriate volume(s) of ChIP dilution buffer (0.01% SDS, 1.1% Triton X- 100, 1.2mM EDTA, 16.7mM Tris-HCl, 167mM NaCl, pH 8.1) were/was then added to make the DNA concentrations of all samples equivalent.

75µl of salmon sperm/protein A agarose slurry (Sigma-Adrich, USA) was added and the mixture was then rotated for 30 minutes at 4°C followed by centrifugation at 10,000×g for 1 minute at 4°C. The resulting pellet was then washed for periods of 5 minutes each with different buffers at 4°C in the following order (except for the Tris-EDTA (TE) buffer washes, which were carried out at room temperature): low salt immune complex buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl), high salt immune complex buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl, pH 8.1), lithium chloride buffer (0.25M LiCl, 1% IGEPAL NP40, 1% deoxycholic acid (sodium salt), 1mM EDTA, 10mM Tris Hcl, pH 8.1) and twice with TE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA, pH 8.1). After

each wash, a 1000×g centrifugation step was carried out and the resulting supernatant was discarded. After the 2nd TE buffer wash, the pellet was transferred to a 1.5ml tube (Eppendorf, Germany). Pellets from the washes and inputs were then suspended in 10% Chelex-100 resin. This mixture was then boiled at 95°C for 10 minutes and allowed to cool briefly. 1500 units of proteinase K (Fluka, Germany) were then added and the suspension was heated at 55°C for 30 minutes which was followed by a boiling step at 95°C. The sample was then put on ice or stored at 80°C.

2.3.8. ChIP PCR detection of bound hTERT gene fragments

The following primers were used to amplify a segment of the hTERT gene promoter: Forward primer: 5'-GGCCGATTCGACCTCTCT-3', reverse primer: 5'-CGGAGCTGGAAGGTGAAG-3'. PCR reactions consisted of 15/30µl of input/ChIP sample, 1.25 units Truestart *Taq* polymerase (Fermentas, Canada), 1×Truestart buffer, 750µM $MgCl_2$, 500nM primers, 50mM dNTPs and nuclease-free dH2O up to a final reaction volume of 50µl. The PCR cycles were as follows: 2 mins at 95 °C followed by 40 cycles (45s at 95 °C, 30s at 72 °C and 45s at 64°C). Products were subsequently resolved on a 1.5% agarose 1×TAE gel stained with ethidum bromide. Samples were mixed with 6×bromophenol blue dye and then loaded. The gel was run at 100v for 1 hour and products were visualised with Fujifilm image reader Las-3000 (Fujifilm Photo Film Co., Japan).

2.3.9. Vectors and plasmids used in luciferase assay

The pLUC-330 vector used in this study was kindly provided by Silvia Bacchetti (McMaster University, Ontario) and has been previously described (Cong et al., 1999). pLuc330 was originally generated by cloning a fragment of the hTERT promoter in the multiple cloning site of a pGL2-enhancer plasmid. To generate the pLUC plasmid that lacked any hTERT segment, pLUC-330 was digested with the *Xma1* restriction enzyme (Fermentas). The products of the digestion were then resolved in a 1% agarose gel in 1×TAE buffer. The plasmid was purified from the gel with the Qiaquick gel extraction kit (Qiagen, Germany) as per guidelines specified by the manufacturer. The linearised plasmid (lacking the hTERT promoter segment) was then religated to generate pGL2-enhancer vector lacking the hTERT promoter fragment, which was thereafter referred to as pLuc. To increase the respective quantities of pLuc and pLuc-330, they were transformed into chemically competent Top10 E.coli (Invitrogen, USA) following the manufacturer's protocol. Successfully transformed cells (selected by culturing on LB agar containing ampicillin were then inoculated into a 250ml Luria Broth culture and incubated overnight. E.coli were then lysed and plasmids were extracted using a Qiafilter Plasmid Maxi kit (Qiagen, Germany) in accordance with the manufacturer's instructions.

2.3.10. Luciferase plasmid transfection and Relative Luciferase assay

Cells were seeded at densities of 5×10^4 cells per well on a 24-well plate (Sarstedt, Germany). Transfection was performed on the following day, when cells were 90-100% confluent. For each well, depending on the plasmid and siRNA to be added, a 50µl total volume Opti-MEM (Gibco, Germany) solution was prepared which consisted of: 50ng

Renilla luciferase (as an internal transfection control), 5μg pLuc330/pLuc and 0.1nM Scr/Ets2/c-Myc and RuvBl2 siRNA. This solution was left on the bench for 5 minutes at room temperature. An equal volume of Opti-MEM containing 4 %(v/v) Lipofectamine 2000 (Invitrogen, USA) was then added to this solution and the resulting transfection complex was then left on the bench at room temperature for 20 minutes. The transfection complex was then added to each well and incubated under cell culture conditions as previously described for 6 hours. At this point, the transfection agent was then removed and replaced with 500μl D-MEM medium.

Luciferase assays were carried out using a Dual-Glo luciferase assay system (Promega, USA) in accordance with the manufacturer's protocol. Briefly, cells were harvested and lysed by adding 100 μl passive lysis buffer to each well. 10 μl of this lysate was then removed to a luminometer tube. 50 μl of Dual-Glo luciferase substrate was added and firefly luciferase activity was measured using a luminometer. An equal volume of Dual-Glo stop and substrate (which consists of a stop solution that inhibits the firefly luciferase-substrate reaction the substrate for renilla luciferase) was then added and Renilla luciferase activity was measured. Luciferase readings were expressed as firefly luciferase activity as a fraction of Renilla luciferase activity. Transfections and subsequent luciferase measurements were carried out in duplicate in three replicate experiments.

2.3.11. Semi-quantitative TRAP assay

Cells were transfected with siRNA constructs in the manner described in section 2.3.4. The telomerase PCR enzyme-linked immunosorbent assay (Roche Diagnostics,

Germany) was used to measure telomerase activity, following the manufacturer's protocol. 2×10^5 cells were transferred to an eppendorf tube and cells were pelletted by centrifuging at 3000×g for 10 minutes at 4°C. Cells were then washed once with PBS. Cell extracts were prepared by adding 200µl lysis agent to the resulting pellet and this was left on ice for 10 minutes. The lysate was then centrifuged at 16,000×g for 20 minutes at 4°C and 175µl of it was then transferred to a new tube. For PCR, 3µl of the lysate and 25µl of the reaction mixture (containing 2xTris-EDTA (TE) buffer, biotin-labelled P_1-T_s and P_2 primers) were combined and made up to a total volume of 50µl with nuclease-free water in a PCR tube. Reaction tubes were first heated at 25°C for 30 minutes (to facilitate primer extension) and then telomerase was inhibited by heating to 94°C for 5 minutes. Product amplification consisted of 30 PCR cycles. Each cycle was comprised of the following steps: 94°C for 30s, 50°C for 30s and 72°C for 90s. The PCR was concluded by heating the reaction at 72°C for 10 minutes. 5µl of the amplification product was added to 20µl of denaturation solution and incubated at room temperature for 10 minutes. 225µl of hybridization agent was then added and the mixture was vortexed. 100µl was then transferred to a well of a microplate module precoated with anti-digoxigenin antibody and incubated at 37°C for 2 hours with shaking at 300rpm. The well was rinsed with wash buffer. 100µl of anti-DIG-POD was added and then it was incubated at 25°C for 30 minutes with shaking at 300rpm. For colour development, 100µl 3, 3', 5, 5'-tetramethylbenzedrine was added to the well and was incubated at room temperature while shaking at 300rpm. The reaction was then terminated by addition of 100µl of stop reagent. Absorbances were read at 450nm and 690nm wavelengths by a microplate reader (Biotek, USA). Telomerase activity was determined by subtracting

A_{690nm} from A_{450nm} for each sample. A negative control was prepared by treating 5µl of cell lysate with 1µl of RNAase. Samples are considered to be telomerase-positive if the value for samples minus the value for the negative control was greater than $0.2(A_{450nm})$-A_{690nm}.

2.3.12. Immunohistochemistry

Specimens were initially immersed in a number of solutions in the order displayed in table 2.4.

Table 2.4. List of steps in initial phase of immunohistochemistry.
Period of time is measured in minutes. I.M.S- Industrial methylated spirit. PBS-Phospho-buffered saline. PBSt-PBS containing 0.05% (v/v) Tween-20.

Step	solution	Period (min)	No. of times
Deparafinization	Xylene	3	2
Rehydration	100% I.M.S.	3	1
	70% I.M.S.	3	1
Wash	PBS	5	2
Peroxidase depletion	3% H_2O_2	10	2
wash	dH_2O	5	1
	PBSt	5	1

Antigen conformation was restored by heating in Tris-EDTA buffer (10mM Tris base, 1mM EDTA, 0.05% Tween-20) for 15 minutes. The specimen was then given 2×5 minute washes with PBSt. For antibody staining, the Vectastain ABC kit was used (Vector Laboratories, USA). Potential non-specific antibody sites were blocked by

covering with PBS solution containing 1.5% (v/v) goat serum for 90 minutes at room temperature. Excess blocking solution was then removed. The specimen was then covered with the appropriate 1° antibody diluted in PBS (RuvBl2-1:400, hTERT-1:100) at for 1 hour at 4°C. This was followed by 3×5 minute washes with PBSt. The specimen was then covered with rabbit/mouse 2° antibody solution (PBS containing 0.5% (v/v) rabbit/mouse 2° biotinylated antibody, 1.5% (v/v) goat serum) and incubated at 4°C for 40 minutes at 4°C. The specimen was given 2×5 minute washes with PBSt and 1×5 minute wash with PBS. The AB solution (containing avidin and the biotinylated HRP enzyme; Vector Laboratories, USA) was prepared according to the manufacturer's guidelines and was used to cover the specimen for 30 minutes at room temperature.

This was followed by 3×5 minute washes in PBS. The sample was then covered with prepared SigmaFast 3, 3'-diaminobenzidine (DAB) solution (Sigma, USA) until colour developed. The DAB reaction was stopped by immersing the specimen in dH_2O for 5 minutes. Counterstaining of the tissue was then performed by covering it with haematoxylin for 3 minutes. It was then washed in continuously circulating dH_2O for 5 minutes and then given a 1×5 minute wash with PBS. Specimens were dehydrated by treatment with 70% IMS once for 3 mins, twice with 100% IMS for 3 mins, and were then immersed in xylene twice for 3 mins. Slides were then allowed to dry out. They were then mounted with DPX and coverslip. Microscopic examination was later carried out.

Chapter 3

Using SILAC to isolate regulators of telomerase

3.1. Introduction

Owing to the prevalence of telomerase activity as a characteristic in cancer its regulation is an intensively studied phenomenon. Although much knowledge has been acquired in relation to telomerase modulation relatively little is known about hypothetical 'master' regulators that compel a cell to activate telomerase instead of utilising alternative lengthening of telomeres (ALT) as a telomere maintenance mechanism (TMM) or undergoing apoptosis. The fact that the majority of tumours employ telomerase activation as their TMM would suggest that it confers upon the cell a growth advantage relative to their ALT-utilizing counterparts. Alternatively, the series of molecular events required to trigger telomerase activity may be less complex (and therefore more probable) than for switching on the ALT mechanism. A comparison of the proteomes of two similar cell lines could potentially yield candidates that contribute to the activation of either TMM. A previous study indicated that a more relaxed chromatin structure at the hTERT locus is correlated with telomerase activity whereas a more repressive form is associated with the ALT phenotype (Atkinson et al., 2005). It is therefore possible that a regulator of the chromatin status of the hTERT gene is the key determinant in the commitment of a developing tumour cell to use a particular TMM.

A variety of different experimental approaches have been applied in attempting to discover novel hTERT transcription factors (TFs). In the majority of such studies to date, in silico analysis was initially performed on the hTERT promoter to determine TF binding sites which were subsequently validated (Chen et al., 2008; Zhu et al., 2008). An alternative strategy is to examine TFs that act downstream of a receptor which is

stimulated by an extracellular molecule known to upregulate hTERT expression (Kyo et al., 1999b; Maida et al., 2002; Goueli and Janknecht, 2004). A chemical library screen was exploited to highlight the importance of cyclical E2F-HDAC assembly in repressing the hTERT gene in normal somatic cells (Won et al., 2004). Another more direct TF isolation strategy exploits streptavidin-coated paramagnetic beads (Nordhoff et al., 1999). This TF 'pulldown' procedure facilitates the isolation of proteins bound to a biotin-labelled specific binding sequence that may be implicated in transcription of a particular gene. AP-2β and GRHL2 were discovered to be hTERT-transcriptional activators using a similar procedure (Deng et al., 2007; Kang et al., 2009).

This TF pulldown technique evolved from a DNA affinity chromatography procedure devised more than 20 years ago (Kadonaga and Tjian, 1986). This depends on a concatmerized oligonucleotide sequence that contain a particular binding site and has been used to isolate TFs such as Sp1, AP-1, and Oct-1 (Briggs et al., 1986; Angel et al., 1987; Fletcher et al., 1987; Lee et al., 1987). Another technique used to purify TFs is promoter trapping which involves the binding of these factors to oligonucleotides at nanomolar concentrations (Gadgil and Jarrett, 2002). This has been used to isolate the MafA TF and elements of the preinitation complex at the c-jun promoter (Matsuoka et al., 2003; Jiang et al., 2006b). 2-D EMSA (which involves the interfacing of EMSA with subsequent SDS-PAGE to identify the DNA-binding protein of interest) is another TF isolation procedure that was recently (Stead et al., 2006; Stead and McDowall, 2007). A major advantage of this technique is that it does not require any knowledge of the TFs that bind to the DNA fragment used.

Although such TF purification methods would appear to be appropriate for isolating novel hTERT promoter-regulating TFs, they are prone to a number of technical disadvantages. One example is non-specific binding of proteins to the substrate that is used to immobilize the promoter fragment (Jarrett and Foster, 1995; Gadgil et al., 2001). Another drawback is that these techniques only permit TF isolation in minute quantities, $\sim 10^{-18}$M, which is insufficient for detection by common MS platforms (Jiang et al., 2009). Therefore, numerous difficulties would be incurred in attempting to marry this technique to the SILAC proteomics procedure. By comparing the unpurified nuclear lysates of two isogenic telomerase-positive and -negative cell lines, one takes into account not only TFs, but other proteins that may be contributing to the differential telomerase regulation.

Hypothesis

Two isogenic cell lines that are telomerase-positive and telomerase-negative provide an experimental background that has the potential for the identification of differentially expressed proteins that regulate telomerase activity or are implicated in the ALT mechanism. SILAC provides an experimental framework that could be utilized to identify such proteins that exhibit this altered expression pattern which may be linked to the differential TMM usage.

Objectives

1. The immortalization of a fibroblastic cell type by passaging it beyond crisis. This will result in the generation of cell lines that have overcome replicative constraints due to the acquisition of a TMM.
2. Subsequent characterization of these cell lines to determine whether or not they express telomerase activity or use the ALT mechanism to maintain their telomeres.
3. To determine if there are differentially expressed proteins capable of carrying out DNA strand exchange that could potentially involved in ALT and to subsequently use the SILAC approach to identify such proteins.
4. To use SILAC to contrast the nuclear proteomes of the two cell lines with the aim of identifying differentially expressed proteins that could potentially be implicated in regulating telomerase activity.

3.2 Results

3.2.1. Confirming genomic integration of SV40 genomic sequence in the 6G and 1-3C cell lines

Isolated murine cells in cell culture typically undergo a lesser number of cell divisions than human cells but they do become spontaneously immortalized at a much higher frequency (Tevethia et al., 1998). Human fibroblasts in culture never become spontaneously immortalized, but after SV40 transformation, it occurs at a frequency of 10^{-5} to 10^{-8} cells (Neufeld et al., 1987; Shay and Wright, 1989). The SV40 genome encodes the small and large T antigens, both of which are necessary for cellular transformation (Porrás et al., 1996). However transformation with the large T antigen

alone is sufficient to stimulate further cell divisions beyond the point of senescence and encourages the acquisition of immortality (Jha et al., 1998). The large T antigen promotes cell division by interacting with and inactivating the p53 and Rb proteins that induce senescence/apoptosis when telomeres are sufficiently shortened (Bryan and Reddel, 1994). The intent was to generate immortalized cell lines that would be telomerase-positive and -negative (ALT-positive) and SV40 immortalization procedure was used to this end. Both cell lines would be very similar at the genetic level and therefore suitable resources in a proteome comparison to discover novel TMM regulators.

Fibroblasts were isolated from the jejunum of a male with cystic fibrosis and were then transformed with a plasmid expressing the SV40 large T antigen. This was accomplished by transfecting these cells with the pRSV-T plasmid in which the expression of the SV40 early region of DNA (encompassing the large T antigen) is driven by the 3' LTR derived from the Rous Sarcoma Virus. A single stably-transfected clone emerged which was then cultured until crisis. In order to survive crisis cells must develop a telomere maintenance mechanism as further cell division beyond this phase would reduce genomic stability and prevent cell survivability. Cells began proliferating again implying that they had acquired a means of replenishing their telomere lengths and they were sub-cloned as various cell lines. Southern blotting with a SV40 sequence-specific probe was carried out to show that all these cell lines had pRSV-T stably integrated at the same particular site in the genome (Figure 3.1). This was observed to be the case, indicating that all sub-clones were descended from the same singly-transformed and immortalized cell.

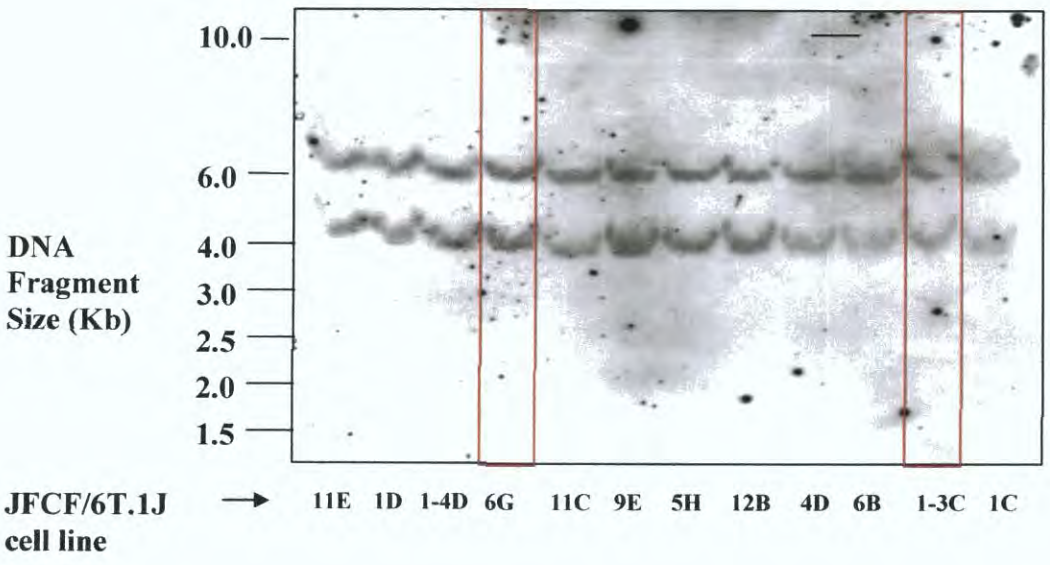

Figure 3.1. A panel of JFCF-6/T.1J subclones are descended from a single cell that was SV40-immortalized.
Southern blot analysis was performed using SV40-specific DNA sequence on digested DNA from the indicated clones indicated derived from immortalised JFCF-6/T.1J cells. The identical bands indicate that the JFCF-6/T.1J cell population is clonal and thus that the subclones shown have ultimately emerged from a single SV40 integration and transformation event in a founder cell. Data were obtained courtesy of Roger Reddel, the Children's Medical Research Institute, Sydney, Australia.

3.2.2. Determining the presence of telomerase activity in the JFCF-derived clones by means of the telomerase repeat amplification assay (TRAP) assay

The JFCF/6T.1J sub-clones had progressed beyond the point of crisis or mortality stage 2 and this implied that they have developed a means of maintaining their telomeres. To determine which of the JFCF/6T.1J-derived clones had activated telomerase their extracts were examined using the TRAP assay. The TRAP assay was first devised in 1994 and since then has served as the standard procedure for the assessment of telomerase activity (Kim et al., 1994). In the TRAP assay TTAGGG-labelled oligonucleotides are combined

with lysates from the cells which one wishes to examine for telomerase activity under appropriate conditions.The reaction product is then resolved on an acrylamide gel. If telomerase is present, a ladder of fragments corresponding to variable degrees of extension by telomerase should be observed. As seen in Figure 3.2, a number of the JFCF-derived clones possessed telomerase activity. Of the two that we selected to investigate further, the 6G and 1-3C cell lines, the former cell line exhibited telomerase activity whereas the latter did not (Figure 3.2).

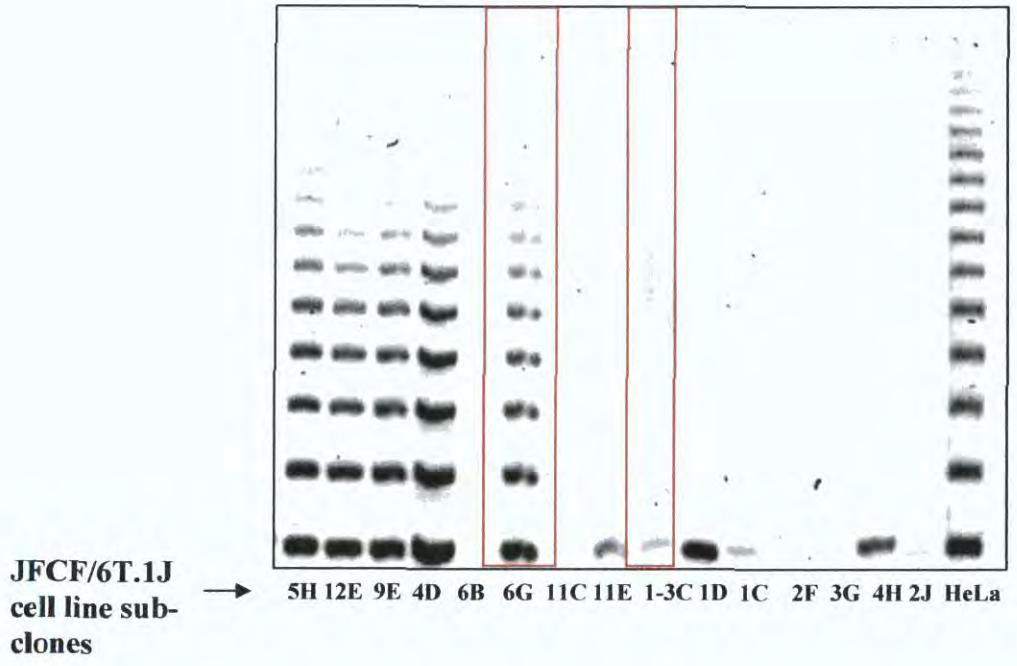

Figure 3.2. TRAP assay to determine presence of telomerase activity.
Clonally derived cell lines from the immortalized JFCF-6/T.1J cell line were examined for telomerase activity using the telomerase repeat amplification protocol (TRAP) assay. A number of cell lines were found to express telomerase activity. In particular, the 6G cell line exhibits telomerase activity whereas the 1-3C cell line does not. In general, a higher percentage of immortalised cells tend not to possess telomerase activity than is the case for clinical cancers (85-90%). Precise reasons for this discrepancy are unknown, but it is possibly due to tighter repression of hTERT gene expression in telomerase-negative cells. Data were obtained courtesy of Roger Reddel, the Children's Medical Research Institute, Sydney, Australia.

3.2.3. Using terminal restriction fragment (TRF) analysis to measure telomere length in JFCF-derived cell lines

The TRF analysis procedure involves southern blotting for the telomere microsatellite repeat using a labeled telomere sequence oligonucleotide sequence as a probe (de Lange et al., 1990). Telomere length is an important indicator of which telomere maintenance mechanism a particular cell line is using. Telomeres in cell lines that employ telomerase as the TMM generally have shorter telomere lengths and have a more uniform distribution (Cesare and Reddel, 2008). This is probably due to the more stochastic nature of telomere extension in the ALT phenotype in two respects: (1) The amount by which a telomere is extended is more variable under recombination-based lengthening process (2) the frequency of extension at a particular telomere is not as perfunctorily regulated as in the case of telomerase activation. As seen in Figure 3.3, the 1-3C cell line is one of the sub-clones which have a greater average telomere length with wider deviation in contrast to that of the 6G cell line which has comparatively short telomeres.

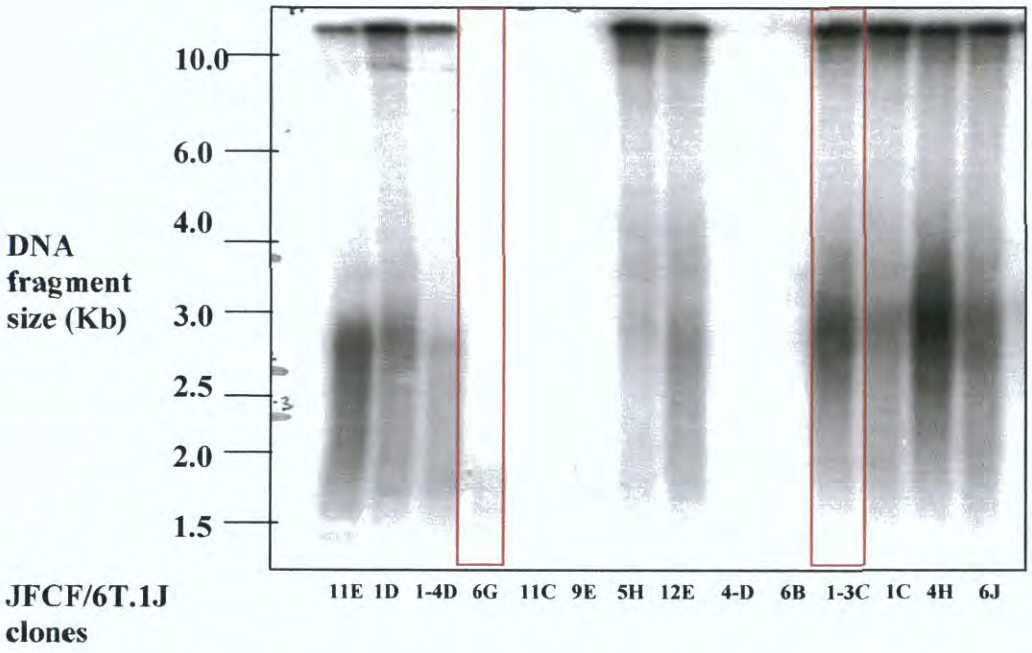

Figure 3.3. TRF length analysis on subclones derived from the JFCF-6/T.1J cells. TRF length analysis, via pulse-field gel electrophoresis, of the indicated clones derived from immortalised JFCF-6/T.1J cells. Telomeric DNA of the 1-3C cell line is long and heterogeneous in contrast to the 6G cell line. This characteristic is typical of cells that use the ALT mechanism to maintain their telomeres.

3.2.4. Using the POM assay to detect differentially expressed proteins which may be implicated in homologous recombination (HR) associated with the ALT process

It was also important to consider the possibility that a differentially regulated protein might also contribute to the establishment of the ALT phenotype in the 1-3C cell line. ssDNA strand pairing and exchange are essential activities in the recombination process. Homologous DNA pairing followed by ssDNA invasion is required for D-loop formation which is one of the transient intermediate structures that are formed during the course of homologous recombination (Pâques and Haber, 1999). HR between telomeres of different chromosomes or between sister chromatids provides two of the mechanisms by which telomere lengths are maintained in cells that utilize ALT as a TMM (Henson et al., 2002).

The pairing-on-membrane (POM) assay was originally devised to detect proteins that can induce homologous DNA pairing and exchange activities that do not require associated exonuclease actvity (Akhmedov et al., 1995). It was later confirmed that one of these discovered proteins is the TLS/FUS oncogene which is known to be capable of carrying out DNA recombination pairing thereby verifying the functional validity of the assay (Bertrand et al., 1999). A POM assay was employed to try and detect such proteins in the nuclear extracts of the 6G and 1-3C cell lines. The POM assay detected a single protein capable of carrying out DNA strand exchange which is more highly expressed in the telomerase-negative, ALT-positive 1-3C cell line than in the 6G and HeLa telomerase-positive cell lines (Figure 3.4).

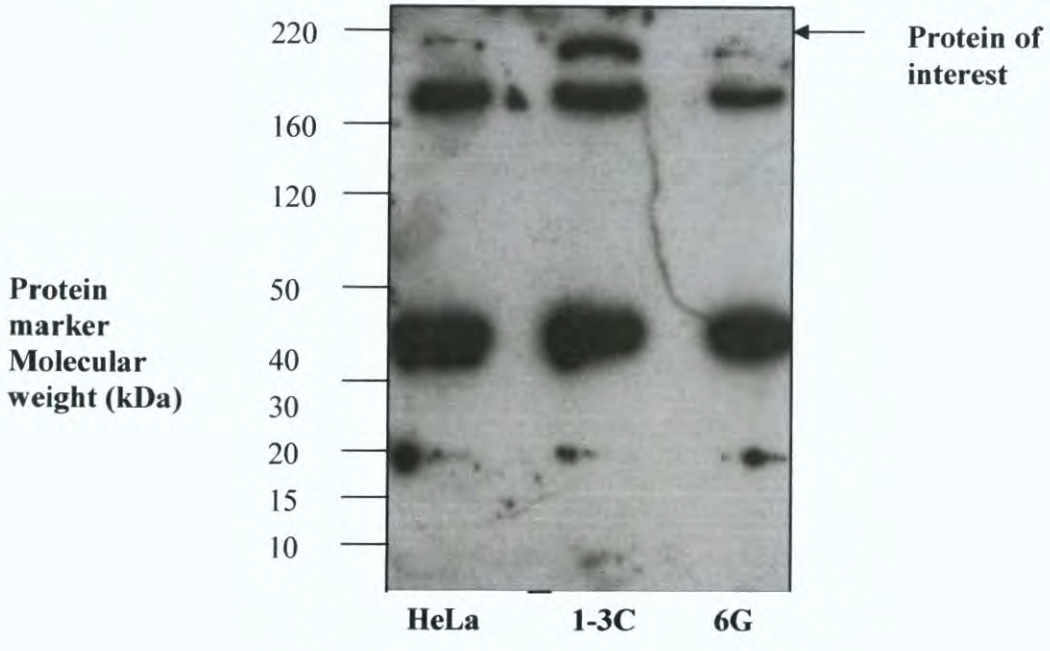

Figure 3.4. Using the POM assay to detect proteins that can facilitate ssDNA exchange in the 6G and 1-3C cells.
The pairing on membrane (POM) assay was peformed on nuclear extracts of the cell lines indicated to detect proteins capable of homologous DNA pairing/exchange activity. A protein that performs DNA stand exchange is more expressed in the 1-3C cell line in contrast to the telomerase-positive 6G and HeLa cell lines.

3.2.5. Using SILAC to identify the differentially expressed protein capable of carrying out DNA strand exchange as detected by the POM assay

The POM assay result indicated that a protein capable of carrying out DNA strand pairing and exchange activities was more expressed in the 1-3C cell line than in the 6G cell line. Electrophoretic migration indicated the protein was in the mass region of 160-220kDa. It was therefore decided to compare levels of protein expression in this mass range by availing of the SILAC technique followed by subsequent MS analysis and quantitation of proteins in this mass range. The 6G cell line was grown in medium supplemented with $^{13}C_6$-lysine that was 6Da greater than the regular ('light') version ($^{12}C_6$) used to supplement the medium for culturing the 1-3C cell line. Nuclear extracts were harvested and combined equal amounts were resolved on a discontinuous gradient SDS-polyacrylamide gel.

In order to compare protein expression both the 6G and 1-3C cell lines, the MSquant computer program was used. To assess the validity of the MSquant software, some of the relative 6G: 1-3C protein quantity ratios were manually checked. This was done by comparing the peak spectral intensities of peptides from both cell lines. One example is the CNELQ peptide sequence corresponding to the SNF2L1 protein (Figure 3.5). The peptide expression ratios calculated in this manner for a particular protein were averaged and then the mean was compared to the value computed by the MSquant algorithm. In most cases the MSquant and manually calculated values were similar. Therefore it was decided to automate quantitation as this software was compatible with data acquired using qTOF mass spectrometry.

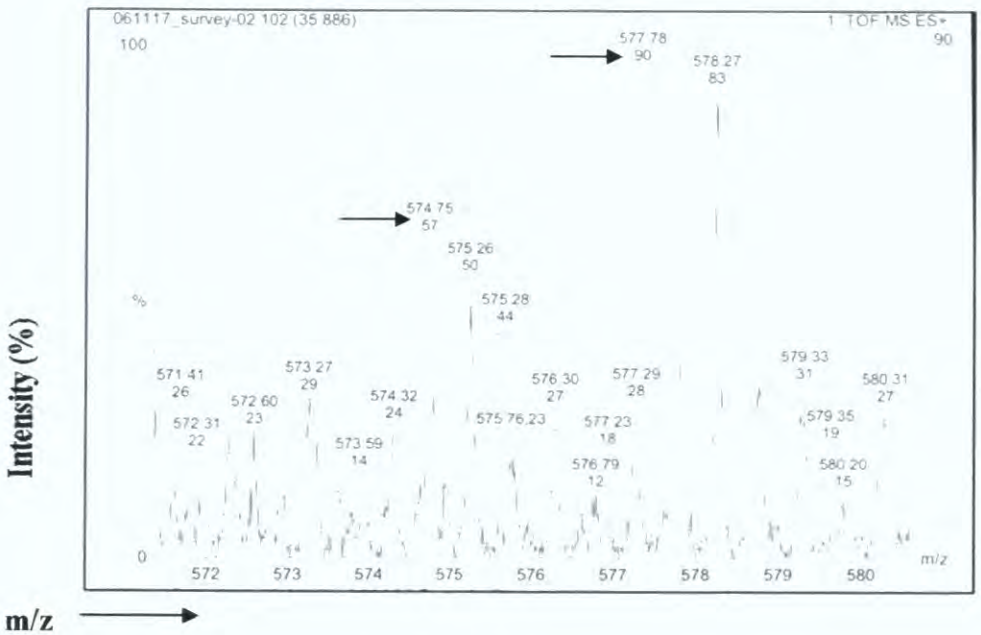

Figure 3.5. Example of comparison of spectral peak intensities to determine peptide expression ratio.
The chromatogram shown is that of the CNELQ peptide belonging to the transcription factor SNF2L1. The peptide with an m/z value of 574.75 came from the unlabelled 1-3C cell line, whereas that with a value of 577.78 came from the 6G cell line that was labelled with $^{13}C_6$-lysine. The peptide is doubly-charged so the m/z difference is approximately 3. Expressing both peak intensities (indicated by arrows) as a 6G: 1-3C ratio gives an expression ratio of ~0.63.

In order to confirm that our SILAC culturing procedure was effective in terms of the percentage labelled amino acid incorporation, a band from the 35KDa region of the gel was removed and subjected to qTOF MS analysis. As seen in Fig 3.6(c), MS spectra of a peptide belonging to the large ribosomal subunit protein L10E show that both peaks have very similar intensity (L/H=1670/1716≈97%). 6 gel slices were excised from the 160-220KDa this region of the gel (as the protein exhibiting homologous DNA pairing activity had a mass in this range) and were then subjected to qTOF mass spectrometry.

MSquant software was used to compare the peptide spectral peak intensities to measure relative protein levels in both cell lines (Mortensen et al., 2010). A fraction of the differentially expressed proteins is listed on table 3.1 (for a full list of proteins quantitated, refer to appendix 1).

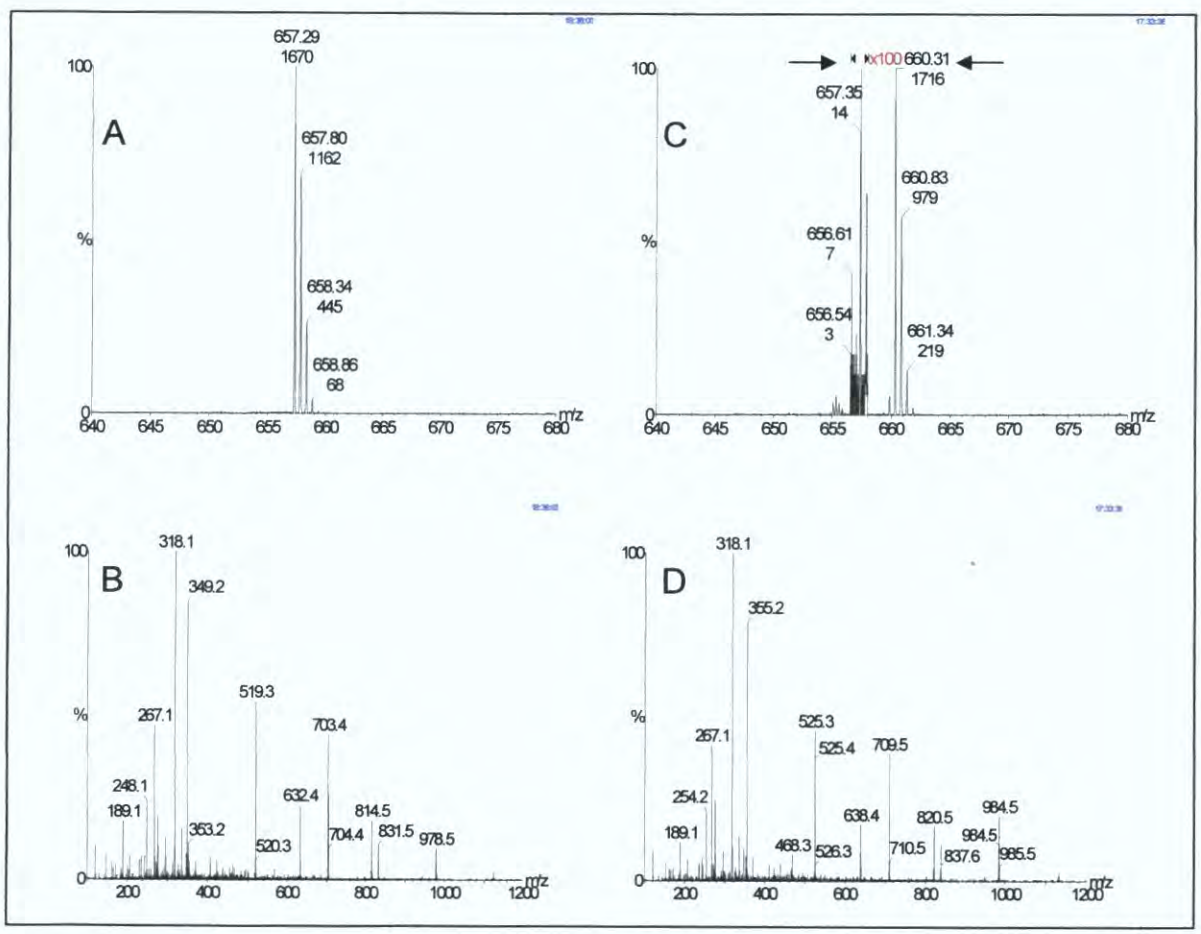

Figure 3.6. Confirming that SILAC labeled amino acid incorporation was 100% effective.

Telomerase-positive fibroblasts were grown in minimal media without lysine that were either supplemented with $^{12}C_6$-Lys (panels A and B) or with $^{13}C_6$-Lys (panels C and D). Cells were harvested, lysed and the extracts were resolved by SDS-PAGE. A band at 35 kDa was cut out, in gel digested with trypsin and the resulting peptides analysed by LC-MS/MS on a Cap LC/QTOF Ultima system. The displayed spectra are mass spectra (panels A and C) of scans containing the tryptic peptide TSFFQALGITTK from the large ribosomal subunit protein L10E and MSMS spectra of the same peptide (panels B and D). Ae indicated by both arrows in C, peak intensities were identical, demonstrating successful incorporation of the labelled lysine amino acid in the 6G cell line.

Table 3.1. The use of SILAC to identify the protein of interest detected in the POM assay.

Telomerase-negative fibroblasts were grown in minimal media without lysine that were supplemented with $^{12}C_6$-Lys and telomerase-positive fibroblasts were grown in minimal media without lysine that were supplemented with $^{13}C_6$-Lys. Nuclear extracts from both cell lines were combined in equal amounts and resolved by discontinuous gradient SDS-PAGE. Six bands (corresponding approximately to the mass region ranging from 160-220kD) were excised, subjected to in-gel tryptic digestion and the resulting peptides analysed by LCMSMS on a Cap LC/QTOF Ultima (Waters, UK) system. The data were then processed using MassLynx 4.0 (Waters). Proteins were searched for by using Mascot software. Quantification ratios were obtained by using MSquant software (developed by Peter Mortensen, University of Southern Denmark, Denmark). The table below displays some of the overexpressed and underexpressed proteins in the 6G (telomerase-positive) cell line relative to the 1-3C cell line.

Description	Accession code	Mass (KDa)	6G: 1-3C ratio
Plectin-1	PLEC1_HUMAN	533.4	2.44
Fact Complex subunit spt16	SPT16_HUMAN	265.85	1.86
Nucleoprotein TPR	TPR_HUMAN	119.84	1.71
ATP-dependent RNA helicase	DHX9_HUMAN	142.2	1.66
DNA-dependent protein kinase catalytic subunit C	PRKDC_HUMAN	473.8	1.66
Uveal autoantigen with coiled-coil domains and ankyrin repeats	UACA_HUMAN	162.4	1.65
Proline-, glutamic acid- and leucine-rich protein 1	PELP1_HUMAN	120.9	1.64
E3 SUMO protin ligase RanBP2	RBP2_HUMAN	362.44	1.53
Mediator of DNA damage checkpoint protei	MDC1_HUMAN	227.69	1.52
Tho complex subunit 2	THOC2_HUMAN	171.16	1.51
eIF-5B	IF2P_HUMAN	139.18	1.25
Dynactin-1	DYNA1_HUMAN	141.6	0.54
Myosin-10	MYH10_HUMAN	346	0.87

Table 3.1 (Continued).

Description	Accession code	Mass (KDa)	6G: 1-3C ratio
SNF2L4 transcription factor	SMCA4_HUMAN	185.1	0.75
Afadin	AFAD_HUMAN	563	0.67
Filamin-B	FLNB_HUMAN	280.2	0.34

3.2.6. Comparing 6G and 1-3C nuclear proteomes using SILAC to identify telomerase regulators

To increase the probability of identifying proteins that were potentially involved in regulating telomerase nuclear extracts were used in carrying out the overall SILAC analysis as most of the currently known key events in telomerase regulation occur in this cellular compartment. Discontinuous gradient gel electrophoresis was used to resolve the proteome to maximize separation for the higher molecular mass proteins (Figure 3.7). 6G cells were doubly-labelled with 'heavy' versions of lysine and arginine ($^{13}C_6$, $^{15}N_4$-arginine and $^{13}C_6$-lysine), while the 1-3C cells were cultured in medium supplemented with regular 'light' versions of these amino acids. In this labelling system, MS analysis would take into account not just peptides with a C-terminal lysine but also those with a C-terminal arginine. Metabolic labelling of cells with heavy isotopes of lysine and arginine leads to complete incorporation into all the trypsinized peptides that represent that comprise the entire proteome (Schulze et al., 2005). Using 2 labelled amino acids allows a wider coverage of the proteome. After resolution by discontinuous gradient SDS-PAGE, 30 gel sections were extracted (see figure 3.7). SILE is an algorithm which can be employed in performing Bruker-HCT mass spectrometry runs which causes

selective acquisition of peak doublets whose relative spectral peak intensity values (Heavy: Light) exceed/are less than 3.5/1.5 in our experiment. This enabled us to concentrate mainly on peptides of proteins that were differentially expressed in both cell lines (in particular those that are highly expressed in the 6G cell line). Comparative levels of proteins from both cell lines are displayed in table 3.2 (the number of peptides used to quantitate each protein can be found in appendix 2).

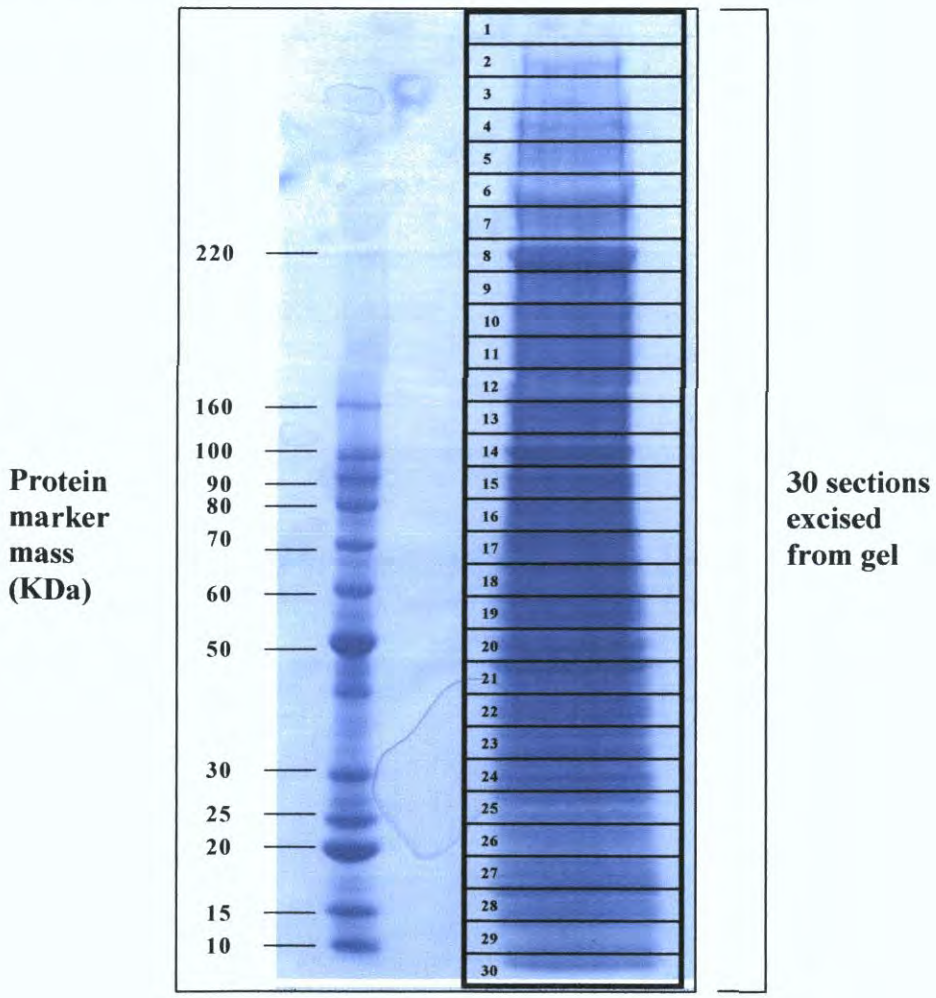

***Figure 3.7.* Proteins for resolved by SDS-PAGE for nuclear proteome SILAC analysis.**
Proteins from both 6G and 1-3C cell lines were extracted and combined in equal amounts. They were then loaded and resolved by discontinuous gradient SDS-PAGE. 30 gel sections were cut out of the gel and then subjected to tryptic digestion for subsequent MS analysis

Table 3.2. The use of SILAC for relative quantitation of the nuclear proteome in 6G and 1-3C cell lines.

Telomerase-negative fibroblasts were grown in minimal medium without arginine and lysine that was supplemented with labelled $^{14}N_4$, $^{12}C_6$-Arg and $^{12}C_6$-lysine. The 6G telomerase-positive fibroblasts were grown in minimal medium without regular arginine and lysine that were supplemented with the respective 'heavy' isotopic amino acids $^{15}N_4$, $^{13}C_6$-Arg and $^{13}C_6$ lys. Cells were lysed and nuclear protein extracts were combined and resolved by SDS-PAGE. Peptides were analysed by LC-MS/MS on a Bruker HCT Ultra ion-trap system. The data were then processed using Proteinanalysis 1.0 software (Bruker). Proteins were searched for by using the X!Tandem software (available at the www.thegpm.org). Quantitation ratios based on spectra were calculated manually. '6G: 1-3C' is the ratio of protein levels in 6G v 1-3C cells.

Protein	Uniprot Acc. code	Mr	6G: 1-3C
RuvBl2	Q9Y230	51.1	3.9
hnRNP A1	P09651	38.6	4.8
HSP-related protein 70kD protein	P54652	70	6.4
hnRNP A1-like protein 2	Q32P51	34.2	5.8
hnRNP A2/B1	P22626	37.4	2.7
Myosin-11	P35749	227.3	1.1
Peripherin	P41219	53.7	1.3
Actin-like protein 3	P61158	47.4	1.1
Ubiquitin-conjugating enzyme E2N	P61088	17.1	0.67
Mitochondrial import inner membrane translocase subunit TIM13B	Q9Y5L4	10.5	2.8
GTFII-I	P78347	112.4	4.2
60S ribosomal protein L11	P62913	20.3	2.9
G-protein-signalling modulator 1	Q86YR5	72.1	1.9
Phosphatidylethanolamine-binding protein	P30086	21	1.3
Myosin-1a	Q9UBC5	118.4	3.3
Signal transducer and activator of transcription 1-α/β	P42224	87.3	4.4
Tubulin-α1 B chain	P68363	50.2	1.7
Succinate dehydrogenase	P21912	31.6	7.4

3.3. Discussion

The results of this chapter indicate that the SV40 genomic region constitutively expressing large T antigen in had integrated into the genome of a JFCF cell and resulted in these cells proliferating beyond the point of senescence and evading crisis. Sub-cloning of the cell lines which had emerged followed by southern blotting of their genomes indicated that all were descended from a single stably transfected cell. Furthermore, 2 sub-populations had diverged, one which used telomerase activation as a TMM and the other which utilized ALT. Two cell lines from each subpopulation (6G and 1-3C) were selected for further analysis. One can envisage two possible scenarios for the evolution of the TMMs in these cell lines: (a) Two of the immortalized cells that descended from the transfected progenitor overcame crisis by using different TMMs or (b) the transfected founder cell initially upregulated telomerase activity to overcome replicative senescence and some of its descendants activated ALT (which repressed telomerase activity) or vice versa.

A number of studies have demonstrated that reactivation of telomerase activity in telomerase-negative cells by means of somatic cell hybridization with telomerase-positive cells repressed ALT activity (Perrem et al., 1999; Ford et al., 2001). However expression of the hTERT component alone does not completely abolish ALT activity, suggesting other factors associated with telomerase activation may be involved (Perrem et al., 2001). There is evidence that SV40 immortalization induces changes which are partially mediated by its large T antigen that are favorable to the upregulation of telomerase activity. SV40 transfection induces the translocation of telomerase to the nucleoplasm where it becomes active (Wong et al., 2002). Moreover, the large T antigen

directly inhibits the activity pRB and p53 tumour suppressors, which are both implicated in hTERT transcriptional repression (Nguyen et al., 1999; Kanaya et al., 2000; Xu et al., 2000).

The Mre11-Rad50-NBS1 (MRN) tripartite complex is involved in HR that is associated with the ALT phenotype (Muntoni and Reddel, 2005). The SV40 large T antigen disturbs the formation of DNA repair sites which involve the Mre11 that is part of the MRN complex involved at HR sites in the ALT phenotype (Digweed et al., 2002). Furthermore, another MRN complex component, Rad50, is also functionally perturbed by the large T antigen (Wu et al., 2004). The p130 protein obstructs telomere lengthening attributed to the ALT phenotype (Kong et al., 2006). The SV40 large T antigen is known to inhibit the activity of p130 (a member of the Rb protein family) by phosphorylating it and therefore may promote ALT via this mechanism (Lin and DeCaprio, 2003). On the basis of this existing evidence, it would appear that an SV40-immortalization followed by stable expression of the large T antigen induces a greater number of molecular events that favour the development of telomerase as a TMM. This hints at the possibility that some telomerase activator/repressor may be downregulated/upregulated in the 1-3C cell line.

The POM assay revealed a protein of ~160-220kDa which performed homologous DNA pairing and exchange and whose levels were much higher in the 6G cell line in contrast to the 1-3C cell line. Searching the Swiss-Prot database revealed no such protein of this approximate mass currently known to possess DNA strand exchange activity. In the MS analysis following the POM assay none of the differentially expressed proteins were capable of carrying out strand exchange based on their known functions. However it was

noted that the levels of the DNA protein kinase catalytic subunit (DNA-PKcs) were more elevated (1.66:1) in the 6G cell line relative to the 1-3C cell line. DNA-PKcs is a subunit that contained in the DNA protein kinase (DNA-PK) complex along with the Ku70-Ku80 heterodimer and stimulates the process of repairing DNA double strand breaks by promoting the machinery to implement non-homologous end-joining (West et al., 1998). DNA-PKcs has also been implicated in protecting telomeres by functioning as an end-cap (Williams et al., 2009). It is possible that the lower levels of DNA-PKcs in the 1-3C cell line in contrast to the 6G cell line may favour the activation of the ALT mechanism instead of telomerase activation.

The MRN complex is associated with a Rad51 protein which is capable of strand pairing and exchange functions but does not have a similar mass (San Filippo et al., 2008). Recently a POM assay followed by a proteomics screen was carried out in lymphoblastoid cells to identify all the proteins that were capable of mediating homologous DNA pairing and exchange (Guipaud et al., 2006). Four proteins were identified exhibiting such activity: TLS/FUS, EWS, hTAF(II)68 and PSF. None of these has a mass similar to the protein highly expressed in the 1-3C cell line in our POM assay. Therefore it is possible that the protein in question is specific to the cell type or perhaps specific to the ALT phenotype itself.

By incorporating the SILE parameters into the mass spectrometry run, the amount of data acquired was biased for differentially expressed proteins. Although none of the proteins identified have been peviously implicated in telomerase regulation, 4 of them are involved in the control of gene expression. Some of the proteins identified in the MS

analyses were less than/exceeded the maximum/minimum threshold values set by the SILAC procedure. One possible reason for this discrepancy for this is the different ways of calculating the peptide quantity ratios. The hnRNPs interact with nuclear pre-mRNAs in the nucleus. They are generally involved in processing them and faciliating their export from the nucleus to the cytoplasm (Dreyfuss et al., 1993). Two members of this family, hnRNP A1-like protein 2 and hnRNP A1 are upregulated in the telomerase-positive 6G cell line. The latter has been previously found to interact with the c-Myc promoter and affect its expression which could, in turn, affect hTERT expression (Takimoto et al., 1993). Interestingly, 2 other members of the hnRNP family, hnRNPs K and D were found to be upregulated telomerase-positive cell lines and bind to the hTERT promoter (Kang et al., 2009). General transcription factor II-I (GTFII-I) is also upregulated in the 6G cell line. GTFII-I interacts co-operatively with USF1 and 2 at E-box sites so this may also make a contribution to hTERT upregulation in the 6G cell line (Roy et al., 1991). A number of other proteins of interest were identified such as ubiquitin ligase E2N (UBE2N), Signal transducer and activator of transcription 1-α/β (STAT1-α/β) and RuvBl2. These proteins are directly/indirectly involved in telomere maintenance and therefore it was decided to investigate these further.

Chapter 4

Validation of selected differentially expressed genes as determined by SILAC analysis

4.1. Introduction

In the previous chapter, the global nuclear SILAC analysis revealed a number of differentially expressed proteins in the proteomes of the 6G telomerase-positive and 1-3C telomerase-negative cell lines. Both cell lines were derived from a single cell and appear to only differ with respect to telomerase activity status. Therefore there is a reasonable likelihood that any detected differences in protein expression could potentially affect/be a consequence of telomerase activity status in each cell line. Validation of selected proteins of interest on the basis of mass spectrometry (MS) analysis identification/quantification is a vital step in any experimental strategy involving quantitative proteomics. Proteins actually identified by MS analysis are only a fraction of the entire pool while those quantified constitute an even smaller proportion of the overall protein complement (Bantscheff et al., 2007). A number of biological factors and technical details pertaining to SILAC analysis may impede the accurate quantification of a protein corresponding to a particular peptide(s). These include ambiguities in peptide-dependent protein identification due to alternative splicing and low protein abundance that obstructs accurate identification/quantification (Nesvizhskii et al., 2007).

Computer algorithms are frequently employed to generate functional inferences from the data available that enable the researcher to concentrate further experimental analysis on a smaller number of proteins (Haoudi and Bensmail, 2006; Ekins et al., 2007; Kumar and Mann, 2009). The incorporation of SILE parameters in conducting the MS run reduced the amount of data to a capacity that alleviated further processing by computationally

intensive data-mining procedure. This allowed us to focus only on proteins of interest. A literature search was conducted to determine, on the basis of previous investigations, if any of our profile of differentially expressed proteins might be implicated in telomerase regulation. We selected three such proteins in this manner and decided to subject them to further investigation: RuvBl2 (upregulated in the 6G cell line), STAT1-α/β (upregulated in the 6G telomerase-positive cell line; hereafter it will be just referred to as Stat1) and UBE2N (upregulated in the 1-3C cell line).

RuvBl2 (RuvB-like helicase 2; also known as reptin, reptin52, tip49b, Tip48, ECP51, Ino80H and Ino80J) belongs to the AAA+ (ATPases associated with diverse cellular activities) family of ATPases. RuvBl2 was originally identified in humans based on its sequence homology to the bacterial RuvB protein which promotes branch migration to facilitate the resolution of Holliday junctions (Kanemaki et al., 1997). The AAA+ ATPases are a sub-group of the of the Additional Strand catalytic E group of the P-loop NTPase family (Snider and Houry, 2008). Along with the Walker A and Walker B motifs characteristic of this superfamily, the AAA+ ATPase module is required for nucleotide binding and oligomerization (Ogura and Wilkinson, 2001). RuvBl2 and its homologous partner, RuvBl1 can both form homohexamer complexes (Gribun et al., 2008; Torreira et al., 2008). RuvBl2 has broad repertoire of functions such as the regulation of gene expression, snoRNP assembly and DNA repair (Jha and Dutta, 2009). In particular, it is transiently implicated in telomerase RNP assembly (Venteicher et al., 2008). Because of this and its range of other functions, it was included in our panel of candidates to be further investigated.

STAT1 is a member of the STAT family of a transcription factors and was first discovered almost 2 decades ago (Kessler et al., 1990; Schindler et al., 1992). The pathway leading to STAT1 activation is usually stimulated by the binding of interferon to receptors, thereby causing transphosphorylation of the two associated JAK tyrosine kinases by each other and their subsequent homodimerization. This permits the binding of STAT1 and its phosphorylation that in turn facilitates homodimerization or heterodimerization with other STAT family members. This dimeric complex then interacts with ISGF3G/IRF-9 and translocates to the nucleus to activate transcription from a promoter harbouring an interferon-sensitive response element (ISRE). Under IL-6 stimulation, STAT1 competes with STAT3 for binding to activated JAKs and reduces STAT3 activity by forming a heterodimer with it (Costa-Pereira et al., 2002). STAT3 is known to contribute to hTERT promoter activation (Konnikova et al., 2005). p53 has been previously identified as a hTERT promoter repressor (Kusumoto et al., 1999; Kanaya et al., 2000; Xu et al., 2000; Shats et al., 2004). Stat1 prevents Mdm2-mediated inhibition of p53 and may therefore also indirectly control hTERT expression through this mechanism (Townsend et al., 2004). These data combined suggest a possible indirect role for STAT1 in hTERT repression.

Ubiquitin-conjugating enzyme E2N (UBE2N; also known as ubiquitin protein ligase N, ubiquitin carrier protein N, Ubc13 and Bendless-like ubiquitin-conjugating enzyme) was was ~2-fold more expressed in the 1-3C cell line. UBE2N forms a heterodimer with the Mms2 protein and in this capacity is responsible for catalyzing the synthesis of non-canonical polyubiquitin chains that are connected by a lysine-63 residue (Hofmann and Pickart, 1999). In yeast the UBE2N-Mms2 heterodimer is necessary for the post-

replication DNA repair pathway (Ulrich and Jentsch, 2000; Xiao et al., 2000). In humans the UBE2N-Mms2 heterodimer, in conjunction with TRAF6, is implicated in IKK activation via ubiquitination which is required for NF-KappaB (NF-KB) activation (Deng et al., 2000). The expression of NF-KB has been linked to hTERT transcriptional activity in previous studies (Sinha-Datta et al., 2004; Wang et al., 2004b). Taking this data into account, it is therefore possible that UBE2N expression may indirectly affect regulation of hTERT transcription via this pathway.

Hypothesis

The SILAC comparison of the nuclear lysates extracted from the 6G telomerase-positive and 1-3C telomerase-negative cells uncovered a number of proteins that were differentially expressed in these two cell lines. Both lines were descended from a single clone and therefore should be epigenetically and genetically similar. Any observed differences in the proteome might potentially contribute to telomerase regulation, which typically involves the hTERT catalytic subunit in particular.

Objectives

1. Validation of selected differentially expressed nuclear proteins of interest (as detected by SILAC) at the mRNA and protein levels
2. To ascertain whether or not there is a correlation between one of the differentially expressed proteins and hTERT protein levels

4.2. Results

4.2.1. Comparison of mRNA levels of selected differentially expressed candidate genes as quantified by the nuclear SILAC analysis

Having determined on the basis of currently existing knowledge which proteins warranted further investigation, their comparative mRNA levels in the 6G and 1-3C cell lines were determined by quantitative reverse transcriptase PCR. This would serve as a means of validating their respective protein expression ratios as calculated using the SILAC proteomics approach. In a comparison of RuvBl2 mRNA levels in all three cell lines, significant differences were not observed ($F_{(2,2)}$=0.128, p>0.1; Fig 4.1(a)). In a comparison of UBE2N and ($F_{(2,2)}$=0.132, p>0.1; Fig 4.1(b)) Stat1β mRNA levels ($F_{(2,2)}$=0.152, p>0.1; Fig 4.1(c)) by means of quantitative RT-PCR in all three cell lines, significant differences were not detected .

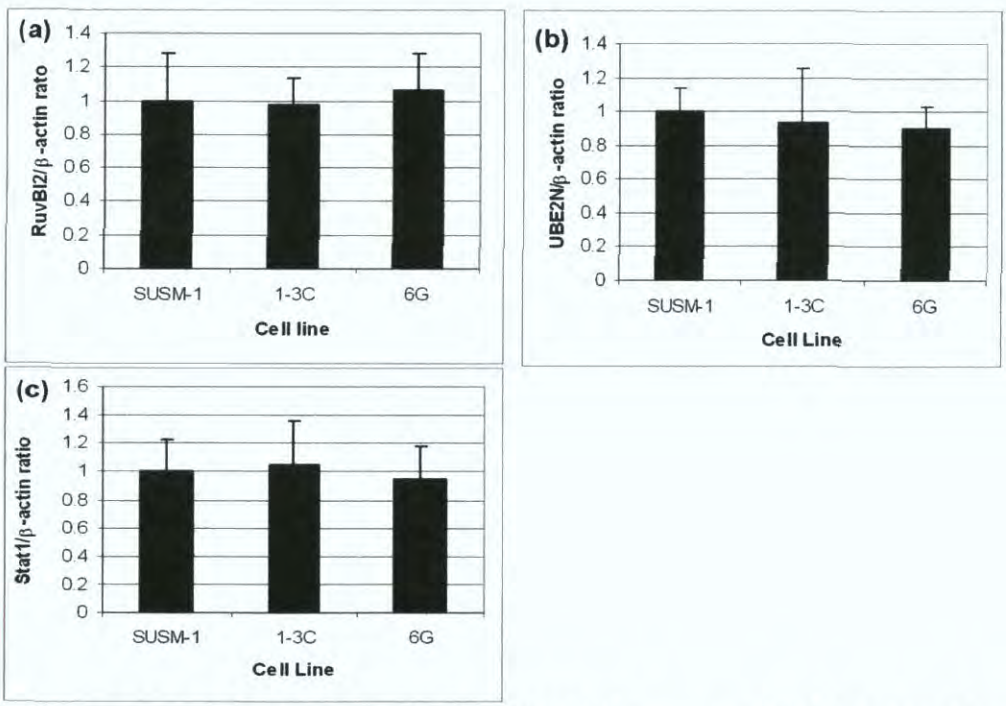

Figure 4.1 Performing Quantitative RT-PCR to assess SILAC ratios of gene expression.

Quantitative reverse transcriptase PCR was performed for 3 of the differentially regulated proteins to determine if the mRNA levels corresponded to the SILAC analysis ratio. The genes RuvBl2 (a), UBE2N (b) and Stat1(c) were examined. No significant differences in mRNA levels between the 1-3C and 6G cell lines for the 3 candidate genes were detected, with each comparison having an F-statistic that corresponded to a p >0.1. Values for the mRNA levels of the 3 genes were normalized relative to a β-actin reference sample. In the graphs displayed, the 6G and 1-3C mRNA levels were normalized relative to that of the SUSM-1 cell line.

4.2.2. Comparing RuvBl2 protein levels in 6G and 1-3C cell lines by western blot analysis

We now sought to validate the relative protein levels for one of the candidates in the 6G and 1-3C cell lines as determined by the SILAC nuclear comparative proteomic analysis. RuvBl2 is a multifunctional protein and is known to participate in many cellular processes such as transcription and the early stages of the DNA repair process (Jha et al., 2009). Although ubiquitous, RuvBl2 is more highly expressed in developing organs or in those which exhibit rapid cell turnover such as in the testis and during induced cardiac hyperplasia (Parfait et al., 2000; Rottbauer et al., 2002). Telomerase activity is required to sustain this high rate of cell proliferation. RuvBl2 is also necessary for telomerase holoenzyme complex assembly. Taking these findings into account, it was decided to further explore the possibility that RuvBl2 might contribute in some degree to its presence in the 6G telomerase-positive cell line.

Western blot analysis was performed to compare RuvBl2 protein levels in 6G and 1-3C cytoplasmic and nuclear extracts. The SUSM-1 and GM639 cell lines were employed as telomerase-negative and -positive controls. In cytoplasmic extracts from all 4 cell lines, RuvBl2 levels were approximately equivalent (see Figure 4.2). However the nuclear RuvBl2 quantity in the 6G cell line exceeded that in the telomerase-negative 1-3C cell line (see Figure 4.2). Interestingly, the level of nuclear RuvBl2 in the GM639 telomerase-positive cell line was also greater than in the 1-3C telomerase-negative cell line (Figure 4.2). Although not as distinct as in the 1-3C cells, there also appeared to be relative underexpression of nuclear RuvBl2 in SUSM-1 cells relative to the telomerase-positive 6G and GM639 cells (Figure 4.2).

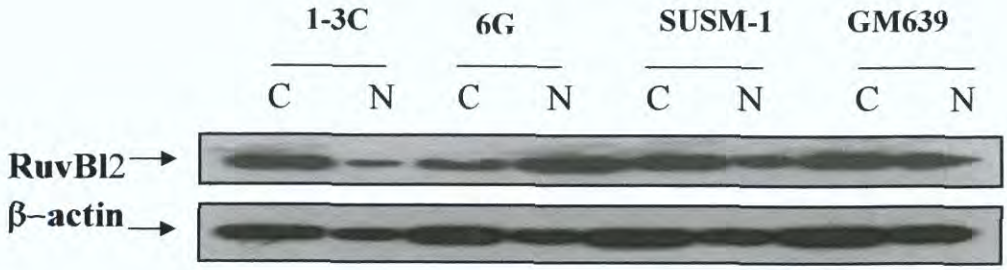

Figure 4.2. Nuclear level of RuvBl2 in the 6G cell line exceeds that in the 1-3C cell line.
Western blotting was performed with nuclear and cytoplasmic extracts with an antibody the for RuvBl2 protein. The extracts from the SUSM-1 and GM639 cell lines were included in the experiment to serve as telomerase -ve and telomerase +ve control cell lines respectively. β-actin was used as a loading control. 'C' and 'N' denote cytoplasmic and nuclear extracts respectively.

4.2.3. The effect of RuvBl2 siRNA knockdown on hTERT protein levels

The previous result suggested a possible relationship between relatively high RuvBl2 protein abundance and the presence of telomerase activity. To assess the putative influence of RuvBl2 on telomerase regulation, transient siRNA knockdowns were carried out to determine if they had any impact on hTERT protein levels. Ets2 is a transcription factor that positively regulates hTERT expression (Dwyer et al., 2007). Serving as a positive control, Ets2 siRNA-mediated depletion induced a partial reduction in hTERT protein levels in the 6G cells (Figure. 4.3). RuvBl2 knockdown in 6G cells by means of transient siRNA transfection also correlated with a downregulation in hTERT protein levels (Figure 4.4.). This experiment was repeated using the HCA-7 telomerase-positive cell line originally derived from a human colonic adenocarcinoma and the reduction in hTERT protein abundance was observed as per the 6G cells (Figure. 4.5).

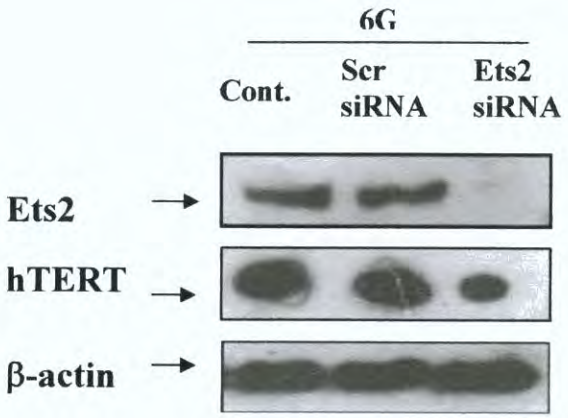

Figure 4.3. Effects of Ets2 knockdown on human telomerase reverse transcriptase (hTERT) expression level.
Ets2 expression was knocked down by siRNA and western blotting was carried out to determine effect on hTERT protein levels. Levels of hTERT protein in the 6G cell line were reduced as a consequence. β-actin was used as a loading control.

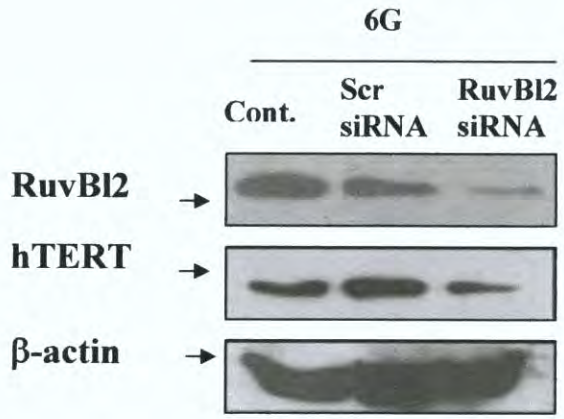

Figure 4.4. Effects of abrogating RuvBl2 expression on hTERT protein levels in 6G cells.
RuvBl2 expression was silenced by siRNA transfection. Western blotting for hTERT was carried out to determine the effect on hTERT expression. β-actin was used as a loading control.

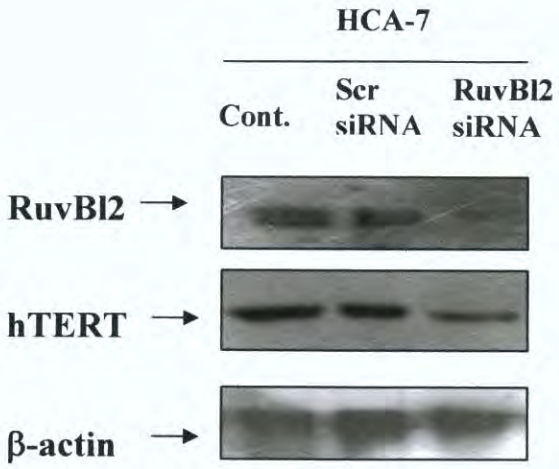

Figure 4.5. Effect of abrogating RuvBl2 expression on hTERT protein levels in HCA-7 cells.
RuvBl2 expression was mitigated by siRNA transfection. Extracts were then analysed by western blotting to determine the effect on hTERT expression. β-actin was used as a loading control.

4.3. Discussion

Quantitative PCR analyses reveal no statistically significant differences in the mRNA levels of the 3 genes RuvBl2, Stat1 and UBE2N ligase that were found by the SILAC quantitative proteomics analysis to be differentially expressed at the protein level. It has been shown that there is a high degree of discordance between mRNA levels and protein abundance such that neither measure can always serve as an accurate estimate for the other. Numerous studies in yeast investigating the relationship between mRNA and protein levels have been performed and illustrate a strong contrast between the expression of both types of biomolecule corresponding to a particular gene (Griffin et al., 2002; MacKay et al., 2004). In mammalian systems, it has been shown that there was a

maximum of 40% correspondence between mRNA and protein levels in human hematopoetic cell lines and in murine hepatic cells (Tian et al., 2004). Another study using lung adenocarcinoma cells estimated a mere 21% correlation in mRNA and protein levels (Chen et al., 2002). One possible reason for the discrepancy in mRNA levels and protein abundance is perhaps due to post-transcriptional regulation which is a more common and sophisticated phenomenon in eukaryotic gene regulation (Mata et al., 2005).

The results in this chapter show an elevated level of nuclear RuvBl2 protein in the 6G cell line in contrast to the 1-3C cell line. Apart from the 1-3C cells, the other cell lines have approximately equal cytoplasmic and nuclear levels of RuvBl2. Therefore, the overall RuvBl2 levels are greater in the 6G cell line than in the 1-3C cell line, which is in accordance with the result obtained by SILAC analysis. Although RuvBl2 carries out the majority of its functions in the nucleus, its distribution between this subcellular compartment and the cytoplasm and the nucleus appears to be variable. In one study using 293T cells, nuclear and cytoplasmic levels of RuvBl2 were equivalent (*Kim et al.*, 2006). It has also been reported that approximately one third of the RuvBl2 pool is nuclear (Gallant, 2007).

Other studies have reported that RuvBl2 presence more is predominant in the nucleus (Holzmann et al., 1998; Gohshi et al., 1999; Sigala et al., 2005). Nuclear RuvBl2 levels were found to be elevated in developing neural progenitor cells (Jaishankar et al., 2009). The RuvBl2 is subject to regulation via nuclear localization. Sumoylation is the addition of small ubiquitin modifier (SUMO) peptide to a protein and is a type of post-translational modification that can influence protein localisation (Geiss-Friedlander and

Melchior, 2007). SUMO is added to K456 of the RuvBl2 protein and is required for its transport to the nucleus (Kim et al., 2006). However, such differential localization is unlikely to account for the differences in nuclear RuvBl2 distribution in the 6G and 1-3 cells, as 1-3 cells do not appear have an excess of cytoplasmic RuvBl2 protein relative to the other cell lines. Considering the relatively high amount of RuvBl2 in the 6G telomerase-positive cell line, we then sought to investigate the nature of the relationship between RuvBl2 and hTERT expression. It was found that knocking down RuvBl2 expression negatively regulated hTERT protein levels. Silencing the expression of Ets2, which is already established as positive regulator of hTERT transcription, exerted a similar effect. This experimental observation was repeated in the HCA-7 cell line, insinuating that the functional relationship between RuvBl2 and hTERT expression is also observed in tumour-derived cell lines.

RuvBl2 has been shown to be a component of a pretelomerase complex that constitutes an intermediate in the pathway for the maturation of the active telomerase complex and is necessary for the snoRNP assembly that confer stability on the hTR non-coding RNA component. Through its interactions with dyskerin and hTERT, hTR RNA stability is affected by its association with the telomerase enzymatic complex and assembly intermediates (Mitchell and Collins, 2000; Mochizuki et al., 2004). However, no existing data supports the notion that hTERT stability is directly influenced by its inclusion as a subunit of the telomerase complex. Therefore, the inhibition of proper telomerase assembly as induced by RuvBl2 downregulation should not have an impact on hTERT protein stability. This would suggest that RuvBl2 may have an additional role in affecting telomerase activity through the modulation of hTERT expression. hTERT regulation has

been shown to occur mainly at the transcriptional initiation stage (Gunes et al., 2000). RuvBl2 is known to play a role in transcription both as a repressor and activator (Gallant, 2007). In summary, the results of this chapter demonstrate that RuvBl2 regulates hTERT protein levels and this effect is potentially mediated modulating its transcription.

Chapter 5

Mechanism of RuvBl2 regulation of hTERT

5.1 Introduction

The previous chapter demonstrated that silencing the expression of RuvBl2 induced a downregulation in hTERT protein levels. hTERT modulation occurs mainly at the transcriptional level. Chromatin remodelling and modifying complexes are required to cultivate a nucleosomal environment that is more conducive to transcriptional activation (Saha et al., 2006; Li et al., 2007). Studies indicate that RuvBl2 is a member of these chromatin-modifying and -remodelling complexes (Gallant, 2007; Sardiu et al., 2008). RuvBl1 and RuvBl2 homohexamers unite to form a dodecameric complex which is itself a component of a number of these chromatin modifiers and remodellers. RuvBl1 and RuvBl2 also act independently of each other, exhibiting opposing functions in a range of cellular processes. For example, RuvBl2 promotes cell growth during zebrafish heart development whereas RuvBl1 inhibits it (Rottbauer et al., 2002). RuvBl1 and RuvBl2 exert antagonistic effects on gene on transcription from the *KAI1* gene promoter as members of distinct coregulator complexes (Rowe et al., 2008).

RuvBl2 along with RuvBl1 have also been identified as constituents of the Ino80 family of chromatin remodellers that partake in transcriptional activation and DNA repair and their stoichiometry relative to other subunits suggests they act as hexameric subunits of a dodecamer (Shen et al., 2000). The human INO80 complex regulates a large number of genes and is recruited to a promoter by the YY1 transcription factor it contains (Affar et al., 2006; Cai et al., 2007). RuvBl2 and RuvBl1 are also subunits of another chromatin remodelling complex which is termed SWI2/SNF2-related CBP activation protein

(SCRAP) (Cai et al., 2005). The overall composition of SCRAP is very similar to the yeast SWR1 complex which is responsible for the insertion of htz1 (the yeast equivalent of the mammalian H2A.Z nucleosomal variant) into nucleosomes to activate transcription (Krogan et al., 2004; Mizuguchi et al., 2004).

The human Unconventional prefoldin Rpb5 Interactor (hUri1) complex acts downstream of the target of rapamycin (TOR) pathway and is known to interact with RNA polymerase II and contains PAF proteins that are implicated in transcription (Yart et al., 2005). The yeast Uri1p equivalent functions in translational initiation (Deplazes et al., 2009). RuvBl1 and RuvBl2 have been identified as components of the Uri1 complex (Gstaiger et al., 2003). The Polycomb group of Genes (PcG) encode for proteins that are assembled into a Polycomb Repressor Complex-1 (PRC1) in Drosophila which induces chromatin compaction to silence gene transcription (Kirmizis et al., 2004). MS analysis of purified PRC1 shows that RuvBl2 is a subunit, but RuvBl1 is not (Saurin et al., 2001).

The Tip60 histone acetyltransferase (HAT) complex composition is variable but is known to contain at least 18 subunits and is involved in transcriptional activation and DNA repair (Sapountzi et al., 2006). Although it is mainly associated with histone acetylation it also acetylates other transcription factors such as the androgen receptor and upstream binding factor (UBF) (Gaughan et al., 2002; Halkidou et al., 2004). As a histone acetyltransferase, TIP60 complex belongs to the MYST (so-called after the MOZ, Ybf2/Sas3, Sas2 and TIP60 founding members) class of histone acetyltransferases RuvBl1 and RuvBl2 have both been defined as members of this complex but are not responsible for its helicase activity (Ikura et al., 2000).

RuvBl2 has been shown to interact specifically with transcription factors that bind directly to DNA. The RuvBl2 yeast ortholog, Rvb2p, has been demonstrated to interact with TATA-binding protein (Kanemaki et al., 1997; Ohdate et al., 2003). It also engages with the NF-κB transcription factor at the *KAI1* gene promoter (Kim et al., 2006). Studies show that RuvBl2 and RuvBl1 interact with c-Myc (Wood et al., 2000; Bellosta et al., 2005). Activating transcription factor 2 (ATF2) regulates a number of genes that are involved in growth, cell cycle progression and counteracting physiological stress. RuvBl2 interacts with ATF2 and inhibits its transactivation capacity (Cho et al., 2001). The E2F1 TF also regulates genes required in the cell cycle and it is known to recruit RuvBl2 (Taubert et al., 2004). In some of these cases, RuvBl2 is likely to interact with the bound TF as a component of the TIP60 HAT complex.

Hypothesis

It has been shown that RuvBl2 affects the levels of hTERT protein. hTERT gene expression is primarily controlled by transcriptional mechanisms. RuvBl2 is known to regulate gene expression through transcriptional regulation. It is therefore proposed that RuvBl2 regulates hTERT expression by modulating its transcription and this subsequently has effects on telomerase activity.

Objectives

Our objectives for this chapter were:

1. To determine if RuvBl2 is recruited to the hTERT promoter by means of ChIP.

2. To examine if downregulation of RuvBl2 can reduce transcriptional activity of the hTERT gene.

3. Given the importance of hTERT transcriptional activation in cancer, it was important to assess the presence and distribution of RuvBl2 and hTERT in colon tumour specimens.

4. To measure telomerase activity and to evaluate whether or not it is reduced under condtions of downregulated RuvBl2 expression.

5.2. Results

5.2.1. Assessment of RuvBl2 ability to interact with the hTERT promoter

In order to assess the possibility that RuvBl2 interacts with the hTERT promoter, the ChIP assay was employed. Although hTERT gene expression is already activated in the 6G cells, they were cultured under EGF treatment to stimulate further recruitment of positive regulators to the hTERT promoter, thereby strengthening any putative signal detected by the ChIP assay. EGF has been previously shown to activate hTERT gene expression in fibroblast cells (Li et al., 2006). Ets2 is stimulated at least partially through the MAP kinase signal transduction pathway to bind to the hTERT promoter and acts in a cooperative fashion to concomitantly stimulate c-Myc interaction with the promoter (Xu et al., 2008a). The ChIP PCR step was performed with primers specifically targeting a region of the promoter that encompasses an established c-Myc-binding E-box (nucleotides -160 to -165) in addition to the adjacent Ets2 binding site (Figure 5.1).

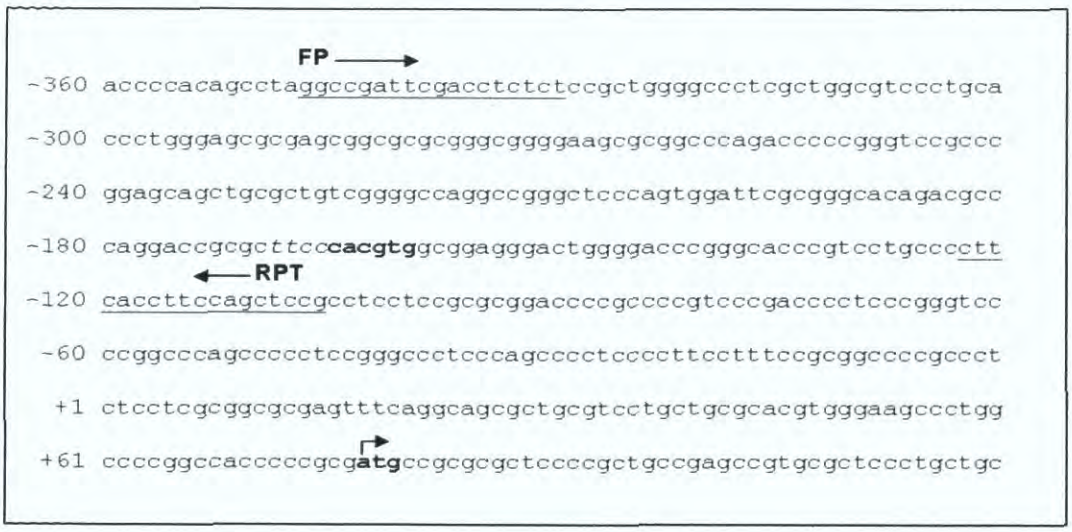

Figure 5.1. Sequence of hTERT promoter encompassed by primers used in the ChIP assay.
Forward primer (FP) and reverse primer template (RPT) sequences are underlined. '+1' refers to the first nucleotide in the template used for transcription as characterised in a previous hTERT promoter sequence analysis study (Takakura et al., 1998). The E-box located from nucleotides -160 to -165 is in bold case with the adjacent Ets2 binding site in italics. The translation initiation codon (in bold) begins at nucleotide +78.

Chromatin was first fixed in vivo by formaldehyde addition to 6G cells in culture. The chromatin was then sheared by sonication in a lysis buffer containing SDS. Protein-DNA complexes were isolated by immunoprecipitation with antibodies specific for Ets2 and RuvBl2. The DNA-protein bonds were then disrupted and the DNA isolated from the bound, immunoprecipitated proteins. Under EGF treatment conditions, it was expectedly observed that Ets2 interaction with the hTERT promoter was increased (fig 5.2). Under the same EGF conditions, RuvBl2 presence was also noticeably enhanced at the hTERT promoter (figure 5.3).

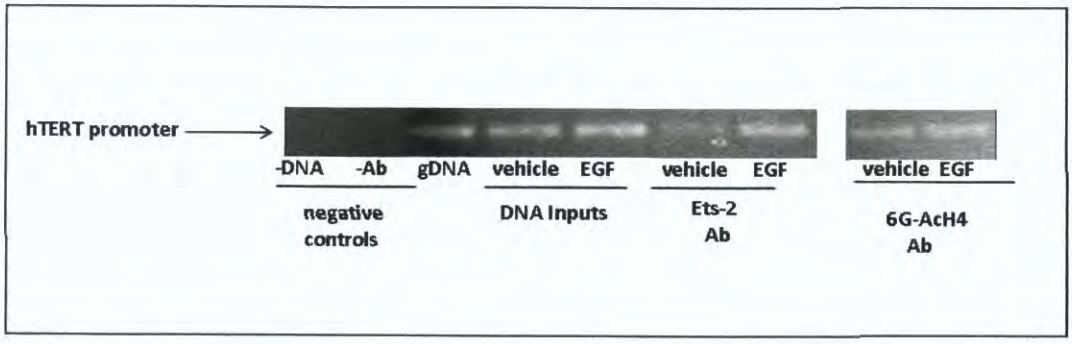

Figure 5.2. Enhanced Ets2 interaction with the hTERT promoter in 6G cells under EGF stimulation as demonstrated by chromatin immunoprecipitation (ChIP) analysis.

6G cells were pre-treated with EGF or control vehicle. These were then fixed with formaldehyde and lysates were incubated with the antibodies for Ets2 and acetylated H4 to capture DNA bound by these proteins. Inputs were used as a loading control. PCR was performed with primers specific for a region of the hTERT promoter (Fig. 5.1). gDNA-genomic DNA.

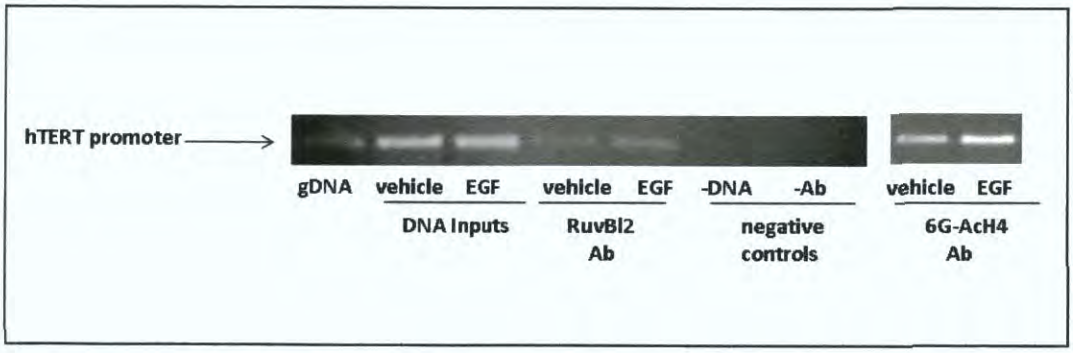

Figure 5.3. RuvBl2 binding to the hTERT promoter *in vivo* under EGF stimulation being demonstrated by ChIP analysis.

6G cells were pre-treated with EGF or control vehicle. These were then fixed with formaldehyde and lysates were incubated with the antibodies for RuvBl2 and acetylated H4 to capture DNA bound by these proteins. Inputs were used as a loading control. PCR was performed with primers specific for a region of the hTERT promoter (Fig. 5.1). gDNA-genomic DNA.

5.2.2. Using the luciferase reporter assay measure the effect of RuvBl2 knockdown on hTERT promoter activity.

Having established that RuvBl2 interacts with the hTERT promoter in vivo by ChIP, we now sought to determine if it regulates the transcription of the hTERT gene. To address this question, 6G and HCA-7 cells were transfected with the reporter plasmid pLuc-330. This construct has previously been used in other studies to ascertain key regulatory elements of the hTERT promoter (Cong et al., 1999). pLuc-330 is a modified pGL2 enhancer plasmid that contains the hTERT promoter sequence from 330bp upstream of the translation initiation codon to 37bp into the second exon linked to the firefly luciferase coding sequence (see figure 5.4). This part of the vector harbouring the hTERT sequence encompasses the 181-bp core promoter region that constitutes the minimal sequence necessary to induce transcriptional activation of the hTERT gene as previously defined (Takakura et al., 1999).

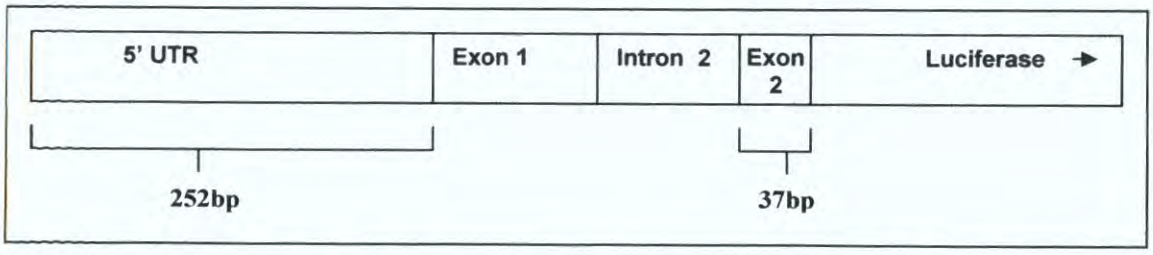

Figure 5.4. Diagram of the hTERT gene segment upstream of the luciferase coding region in the pLUC-330 vector.
The first nucleotide of corresponds to position +1. The hTERT fragment in pLUC-330 encompasses 330bp upstream of the initiation codon, exon1, intron1 and the first 37bp of exon 2.

6G and HCA-7 cells were transiently co-transfected with the pLuc-330 vector and RuvBl2/Ets2/c-Myc siRNA. In 6G cells, RuvBl2 depletion reduced luciferase activity to levels that are comparable with those of the empty control vector, pLuc which lacks the hTERT promoter sequence (see figure 5.4). A similar effect was observed when c-Myc expression was silenced (see 5.4(b)). In HCA-7 cells these observations were recapitulated with RuvBL2 depletion decreasing luciferase activity almost to the same extent as the c-myc expression knockdowns and (see Fig.5.5 (a) and (b)). In both comparisons with Myc siRNA-mediated repression, RuvBl2 downregulation did not appear to diminish luciferase activity to the same extent (see Fig.5.5 (b) and Fig 5.6 (b)).

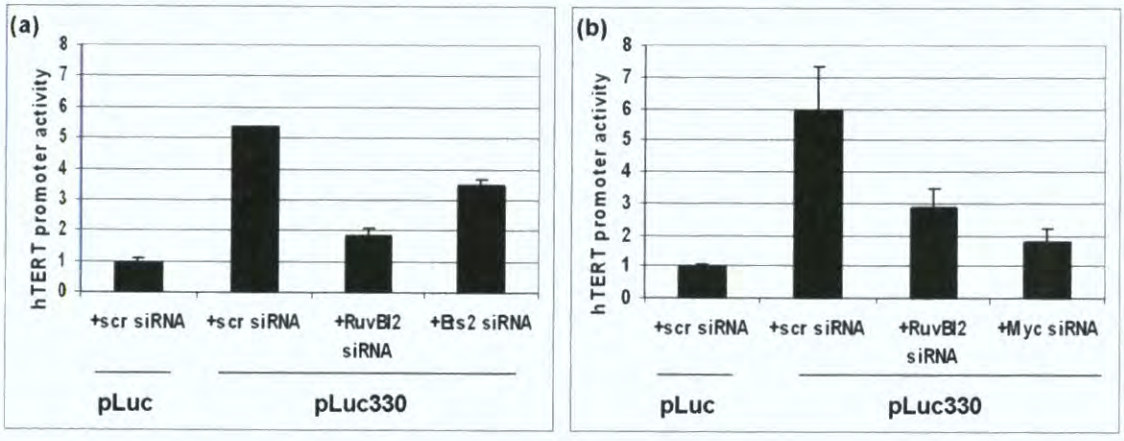

Figure 5.5. Determing the effect of knocking down RuvBl2 expression on hTERT promoter activity relative to Ets2 and Myc knockdowns in 6G cells.

(a) compares the effect of RuvBl2 and Ets2 knockdowns on hTERT promoter activity.
(b) compares the effects of RuvBl2 and c-Myc knockdown. The pLuc-330 construct is a pGL2 enhancer plasmid that harbours 330bp of the core hTERT promoter linked to a firefly luciferase construct. In pLuc the hTERT fragment has been excised. Readings were obtained by calculating a renilla:firefly luciferase activity ratio and expressing it as a fraction of the reading obtained for the cells transfected with control siRNA and pLuc.

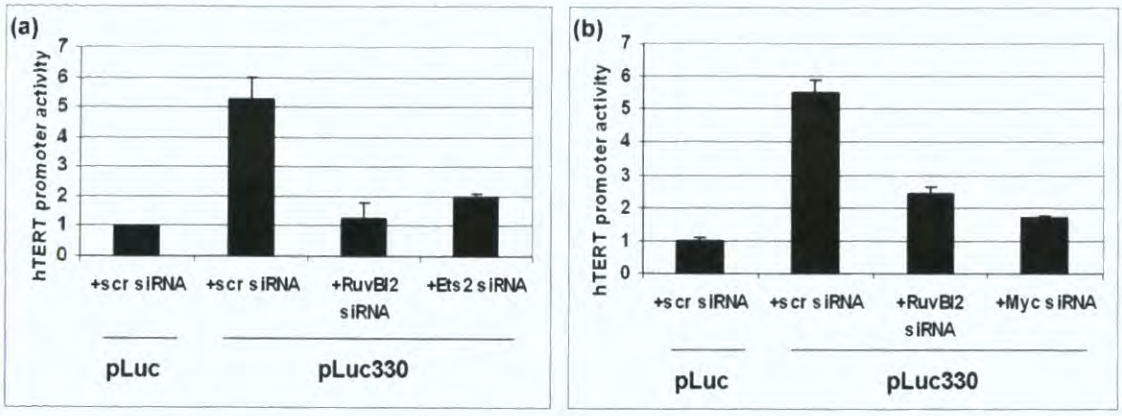

Figure 5.6. Determining the effect of knocking down RuvBl2 expression on hTERT promoter activity relative to Ets2 and c-Myc knockdowns in HCA-7 cells
(a) Compares the effect on hTERT promoter activity of RuvBl2 and Ets2 knockdowns and (b) compares the effects of Ruvbl2 and c-Myc knockdown.

5.2.3. Using immunohistochemistry to assess presence and distribution of RuvBl2 and hTERT in colon cancer

Thus far, our results indicated that RuvBl2 was responsible for maintaining hTERT protein levels and ensuring telomerase activity in the HCA-7 colorectal cancer cell line. We therefore desided to examine the abundance and distribution of RuvBl2 in a preserved tissue sample derived from a colon cancer patient. Haematoxylin staining was performed on preserved paraffin-embedded specimens of colon tumour to determine the subcellular localisation properties of RuvBl2 and hTERT. Positive staining was detected for the RuvBl2 protein in the cytoplasm and the nuclei of most cells (Fig. 5.7(a)). This observation was repeated for the hTERT protein, with some nuclei staining particulalry strongly (Fig. 5.7(b)).

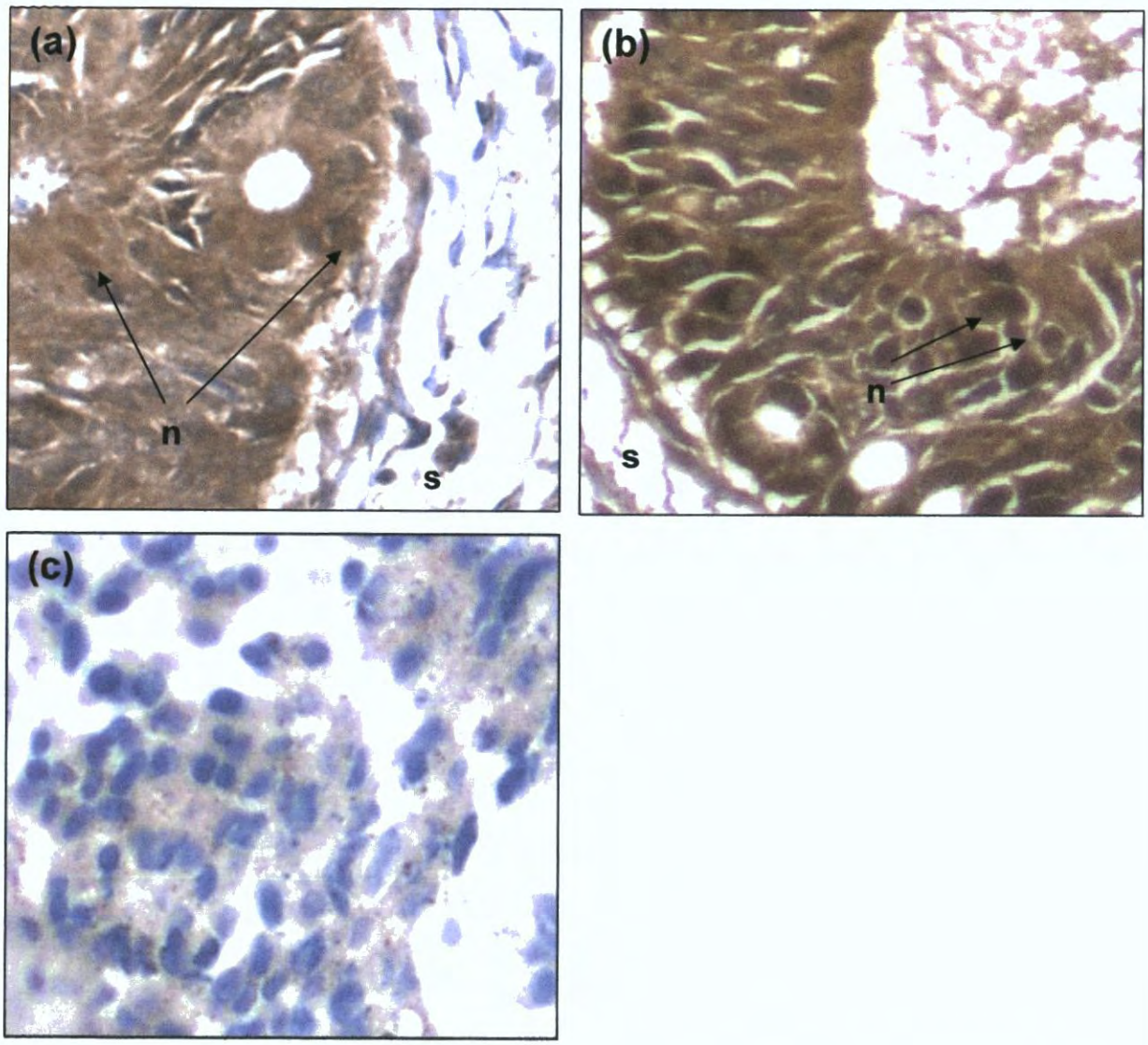

Figure. 5.7. Immunohistochemical detection of RuvBl2 and hTERT in colon tumour samples.

Immunohistochemical staining for RuvBl2(a) and hTERT (b) in human colon cancer cells. (c) is an unstained negative control. Images displayed were magnified 200X. s indicates regions of stroma. 'n' indicates areas of strong nuclear staining.

5.2.4. Determining the effect of RuvBl2 expression knockdown on telomerase activity

The diminution of RuvBl2 expression in 6G cells caused a corresponding decrease in hTERT protein levels. hTERT is an essential component for the reconstitution of telomerase enzyme activity (Weinrich et al., 1997). Thus, a reduction in hTERT levels should result in abrogating the formation of fully functional active telomerase complexes. Therefore reducing the levels of RuvBl2 could potentially mitigate telomerase activity. To examine this possibility, a semi-quantitative TRAP assay was employed which permits a relative determination of telomerase activity. The HCA-7 cell line was used for comparative purposes. 6G, 1-3C and HCA-7 cell lines were transfected separately with Ets2 and RuvBl2 siRNA constructs. The extracts were then harvested and telomerase activity was measured using the semi-quantitative TRAP assay.

In analysing telomerase activity in all 12 extracts as shown in Fig 5.8, statistically significant differences were observed ($p<0.001$). Transfection of RuvBl2 and Ets2 siRNA constructs into 6G and HCA-7 cell lines resulted in reduced telomerase activity in comparison to controls of both cell lines. As expected, telomerase activity in the 1-3C cell line was virtually negligible for all transfections. Interestingly, there was a significant difference in the reduction of telomerase activity brought about by the knockdown of RuvBl2 expression in both 6G and HCA-7 cell lines versus the knockdown of Ets2 expression in both cell lines, with RuvBl2 siRNA transfection inducing a greater reduction in telomerase activity (Fig 5.8).

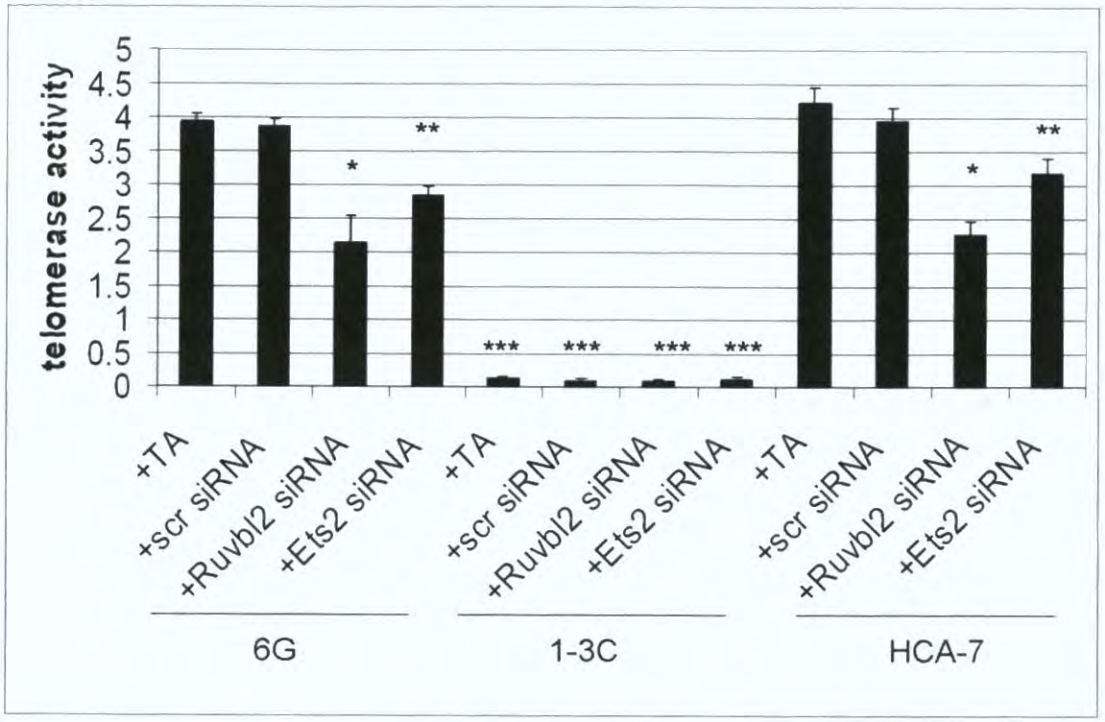

Figure 5.8. RuvBl2 induces a reduction in telomerase activity in 6G and HCA-7 cells. The Roche semi-quantitative TRAP assay was used to assess telomerase activity. siRNA-mediated depletion of RuvBl2 and Ets2 significantly reduced telomerase activity in 6G and HCA-7 cell lines compared to respective controls (ANOVA F-statistic corresponded to $p<0.001$). Samples that have the same number of asterisks above their respective columns are not significantly different from each other based on a Tukey value comparison. TA=transfection agent.

5.3. Discussion

Our ChIP results suggest that RuvBl2 interacts with the hTERT promoter in the 6G cell line. EGF induces the binding of Ets2 to the hTERT promoter via the activation of the MAP kinase pathway (Maida et al., 2002). Our results (Figure 5.2) support this observation. It has been demonstrated that RuvBl2 and its paralog RuvBl1 interact with the c-Myc transcription factor and are necessary for its oncogenic capability (Wood et al., 2000). Ets2 is known to upregulate c-Myc transcriptional activity (Roussel et al., 1994;

de Nigris et al., 2001; Klappacher et al., 2002; Zaldumbide et al., 2002). Ets2 acts in a cooperative manner to promote c-Myc binding to the hTERT promoter (Xu et al., 2008a). Both of these events potentially stimulate c-Myc interaction with the hTERT promoter, inducing an increase in the presence of RuvBl2 at the promoter as detected by ChIP.

Even though RuvBl2 is a member of the Ino80 complex, its interaction with an Ino80-responsive promoter was not previously detected by ChIP in yeast (Jónsson et al., 2004). However, ChIP studies in human cells indicate that RuvBl2 interaction does occur with promoters that require the Tip60 HAT complex for activation (Frank et al., 2003; Taubert et al., 2004). It is possible that differences in the overall subunit arrangement of various coregulatory complexes of which RuvBl2 is a constituent may influence its detection by means of ChIP. Alternatively, RuvBl2 may be transiently associated with the Ino80 complex to facilitate its multimeric assembly and may not be essential for its actual DNA interaction of transcriptional activation functions.

The hTERT-reporter luciferase assay experiments clearly showed that transfection of RuvBl2 siRNA in 6G and HCA-7 cells induced a sharp decrease in promoter activity, as did knocking down expression of the c-Myc and Ets2 hTERT transcription factors. For the RuvBl2 knockdown, a reduction in hTERT promoter activity occurred in both HCA-7 and 6G cells, despite their different (fibroblastic and epithelial) backgrounds implying that RuvBl2 may be a ubiquitous participant in hTERT transcriptional regulation. Neither the Ets2 nor the RuvBl2 knockdown generated as large a relative decrease in promoter activity as c-Myc siRNA. c-Myc is known to recruit the CBP/p300 histone acetyltransferase coactivator to the hTERT promoter (Faiola et al., 2005). It is possible

that recruitment of this coactivator by c-Myc may have compensated for the paucity of transcriptional coregulators that require RuvBl2 which are recruited to the hTERT promoter. This idea is substantiated by the diverse array of transcriptional regulators of the hTERT promoter that have been isolated to date thereby resulting in a plethora of mechanisms to maintain transcriptional activity (Cong et al., 2002).

Considering the possibility that RuvBl2 might affect the acetylation status of genes to modulate their transcription, it is important to reflect upon the chromatin structure of the hTERT promoter in this particular reporter construct. Experimental evidence indicates that nucleosomal architecture is dictated to a certain extent by the underlying DNA sequence (Ioshikhes et al., 2006; Schnitzler, 2008). The hTERT promoter has a high GC content and theoretical evidence has been provided that nucleosomes bind preferentially to such sequences (Chung and Vingron, 2009). Therefore, it is not implausible that the chromatin environment at the hTERT promoter region contained within the reporter construct used in this study is similar to that found at the endogenous hTERT locus. Moreover, the effects of deacetylation on inducing hTERT transcription have been demonstrated using a reporter assay (Cong et al., 2000). This suggests that the relationship between histone acetylation (which RuvBl2 may plausibly impact upon as a member of a coregulator complex) and hTERT promoter activity is likely to be preserved under the experimental conditions described.

RuvBl2 siRNA transfection resulted in a ~50% reduction in telomerase activity, as measured by the semi-quantitative TRAP assay. As expected, the Ets2 siRNA transient transfection also induced a reduction in telomerase activity, although to a slightly lesser

degree. Given that our RuvBl2 siRNA knockdown did not induce a total repression of hTERT protein levels (see section 4.4); it is therefore understandable on this basis that telomerase activity was not completely extinguished. These data consolidate the notion that hTERT transcriptional activation can occur via mechanisms that are independent of RuvBl2 involvement.

Proper evaluation of these results warrants an appreciation that RuvBl2 has been characterised as a protein that plays a role in snoRNP assembly (King et al., 2001). Through this function, RuvBl2 participates in facilitating the maturation of the active telomerase complex (Venteicher et al., 2008). It is possible that despite transcriptional abrogation by siRNA transfection, residual RuvBl2 protein transcribed prior to this point may have permitted the formation of active telomerase complexes that partially accounts for the fact that telomerase activity was not completely extinguished. The Ets2 knockdown did not diminish telomerase activity to levels observed in a previous study (Xu et al., 2008a). In telomerase-positive cells of different origins, it is possible that different transcription factors make different contributions to hTERT regulation on a quantitative basis.

The immunohistochemistry results reveal the distribution of RuvBl2 in the nucleus and the cytoplasm of colon cancer cells. Previous studies in hepatocellular carcinoma cells have demonstrated that while RuvBl2 staining is slightly more intense in the nucleus, it is also present in the cytoplasm (Rousseau et al., 2007). RuvBl1, the homologous partner of RuvBl2, is highly expressed in the cytoplasm of colorectal cancer cells (Lauscher et al., 2007). Despite their predominantly nuclear function, proteins of this class exhibit

substantial cytoplasmic staining in tumour tissue. RuvBl2 appears to be very strongly expressed in the colon cancer cells that were stained. The RuvBl2 staining is quite strong in the specimen examined indicating a high level of expression. This agrees with the findings of a previous study in which its overexpression was detected in 18 colon cancers (Graudens et al., 2006). In summary, the results of this chapter provide evidence that RuvBl2 contributes to transcriptional activation of the hTERT gene. Furthermore, the silencing of RuvBl2 expression also likely mitigates telomerase activity through this mechanism, in addition to its functional consequences pertaining to telomerase ribonucleoprotein complex assembly.

Chapter 6

General Discussion

6.1. General discussion

One of the fundamental questions in telomere biology concerns the identities of the molecular cues that induce a developing tumour cell to adopt one telomere maintenance mechanism (TMM) instead of another. A number of studies suggest that repression of the hTERT gene compels a cell to use the alternative lengthening of telomeres (ALT) mechanism (Atkinson et al., 2005; Cairney et al., 2008). The tight repression of the hTERT gene is mainly due to its highly compacted chromatin structure. This impedes access of transcription factors to the hTERT promoter and DNAase hypersensitive sites associated with active transcription are required for gene expression to occur (Wang and Zhu, 2004, 2003). Telomerase-negative cells that use the ALT mechanism can be induced to activate telomerase through the modification of the epigenetic status of the hTERT promoter (Kumakura et al., 2005). In fully differentiated human cells, the hTERT locus nucleosomal environment can be very repressive, whereas this is not the case for the murine TERT gene, which is even transcribed in differentiated cells (Wang et al., 2009). Tumours of mesenchymal origin tend to use the ALT mechanism at a greater frequency than the typically observed 10-15% rate for cancers in general. Human mesenchymal stem cells, lack telomerase activity unlike many other stem cells and it is due primarily to the relatively compacted nature of the chromatin structure at the hTERT locus (Serakinci et al., 2006). A recent study comparing gene expression profiles of telomerase-positive and ALT-positive cells revealed the downregulation of a number of chromatin modification enzymes that promote transcription in the ALT-positive cells (Lafferty-Whyte et al., 2009). It therefore appears that the epigenetic status of the hTERT promoter is a critical factor that influences a cell to employ either of the two TMMs currently known.

Therefore the key factors that govern the fate developing tumour cell in terms of what TMM it will use are those that control the nucleosomal environment at the hTERT locus. From a signalling perspective, Glycogen Synthase Kinase 3 (GSK3) could possibly represent one such 'master' regulator, as inhibition of its activity by chemical means can suppress telomerase activity (Bilsland et al., 2009; Mai et al., 2009). GSK3 in turn affects the expression of a number of transcriptional regulators such as E2F1 and c-Myc which recruit chromatin-modifying regulators. c-Myc could also fulfil the role of being a 'master regulator' as its DNA-binding activity in ALT-positive cells was found to be significantly reduced relative to telomerase-positive cells (Lafferty-Whyte et al., 2009). Myc has been previously shown to activate gene expression that involves the recruitment of this complex (Frank et al., 2003). RuvBl2, along with p400, RuvBl1, TRRAP and the Tip60 histone acetyltransferase (HAT) enzyme are components of the Tip60 HAT complex. RuvBl2 binds the Mycbox II domain of the Myc protein directly or as part of the Tip60 HAT complex. It is therefore possible that RuvBl2 is implicated in c-myc transcriptional activation of the hTERT gene (Figure 6.1). Given that c-Myc activity predisposes a developing tumour cell to telomerase activaion, RuvBl2 levels could therefore represent a factor of significance in determining the TMM to be used in tumour cells to ensure telomere length replenishment.

By demonstrating that RuvBl2 can influence hTERT upregulation, this study adds to the growing repertoire of functions assigned to RuvBl2 which enable it facilitate tumorigensis. Not only does RuvBl2 interact with c-Myc to activate transcription, but its own gene expression is upregulated by this oncogenic TF and may therefore be an important

contributor to its transformative capacity (Wood et al., 2000). RuvBl2 also interacts with the viral E1A oncoprotein (Dugan et al., 2002). The KAI1 (also known as CD82) protein suppresses metastasis and downregulation of this gene in cancers typically causes enhanced cell mobility and reduced cell adhesive properties (Takaoka et al., 1998; Yang et al., 2002; Jee et al., 2003). A complex consisting of RuvBl2 and the Wnt-signalling pathway coactivator interact with the p50-p50 complex bound to the KAI gene promoter repressive its gene expression (Kim et al., 2006; Kim and Baek, 2009).

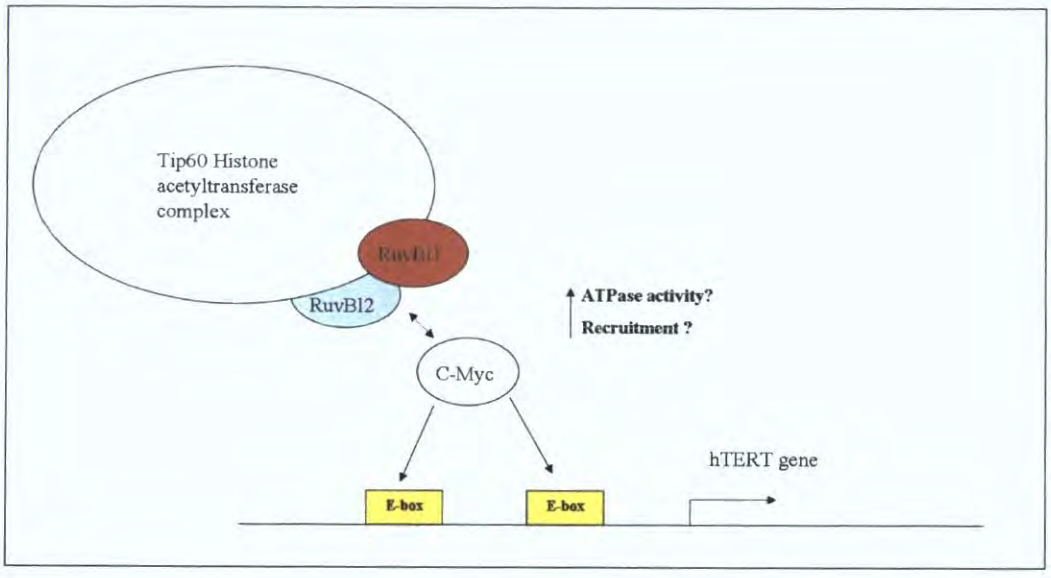

Figure 6.1. Hypothetical mechanism by which RuvBl2 promotes hTERT transcription.
c-Myc recruits the TIP60 histone acetyltransferase to the hTERT promoter, partly through interaction with RuvBl2 and RuvBl1. This results in the histone modifications that enable hTERT transcription. Alternatively, RuvBl2 ATPase activity may be required for chromatin remodelling which is necessary

The histidine triadnucelotide binding protein-1 (HINT-1) acts as a tumour suppressor by regulating the activity of a number of proteins and it prevents apoptosis (Weiske and Huber, 2006; Wang et al., 2007b; Cen et al., 2009). RuvBl1 and RuvBl2 are known to

interact with HINT-1 and it has been hypothesised their binding inhibits HINT-1 activity (Weiske and Huber, 2005; Huber et al., 2008). The activating transcription factor-2 (ATF-2) also acts a tumour suppressor by upregulating the expression of a number of genes that promote apoptosis and its knockdown increases the probability of the emergence of murine tumours (Maekawa et al., 2006, 2008). It is also associated with higher patient survival in cancer (Cheng et al., 2009). RuvBl2 also promotes tumorigenesis by inhibiting ATF-2 activity (Cho et al., 2001). Downregulation of RuvBl2 expression can reduce cell proliferation by obstructing the G2-M cell cycle transition and can induce replicative senescence and it is likely that this is at least partly due to its participation in telomerase holoenzyme assembly (Tyteca et al., 2006; Huber et al., 2008).

A number of studies have highlighted the comparatively high expression levels of RuvBl2 in cancer specimens that may be of clinical significance. RuvBl2 is among the 35 most upregulated genes in hepatoceullar carcinomas in comparison to normal cells infected with Hepatitis-B virus (Iizuka et al., 2006). Another study has demonstrated overexpression of RuvBl2 in 75% of hepatocellular carcinoma specimens and the extent of overexpression is correlated with the grade (severity) of the tumour (Rousseau et al., 2007b). Furthermore, it was also shown that RuvBl2 specifically encourages tumourigenesis by inhibiting apoptosis and promoting anchorage-independent cell growth. It has been reported that RuvBl2 is 2.1-fold more expressed in clinically advanced neuroblastomas that are prognostically unfavourable in comparison to those diagnosed by mass screening which are less malignant (Krause et al., 2005). RuvBl2 is also more upregulated in malignant melanomas than in benign skin nevi or normal tissue specimens (Talantov et al., 2005). RuvBl2 was also found to be overexpressed in a study of 108 bladder tumours (Sanchez-

Carbayo et al., 2006). RuvBl2 and its heterodimeric partner RuvBl1, have also been shown to be highly expressed in colorectal cancer (Graudens et al., 2006; Lauscher et al., 2007).

RuvBl2 has previously been identified as a subunit that is necessary for the assembly of the active telomerase complex (Venteicher et al., 2008). This study revealed that RuvBl2 regulates telomerase activity through an alternative mechanism. In this particular study, it has been demonstrated that it is implicated in the transcriptional activation of the hTERT component. Interestingly, other proteins have been reported to also affect telomerase through multiple mechanisms. One example is the human papillomavirus E6 protein that induces transcriptional activation of the hTERT gene by recruiting E6AP to the hTERT promoter to induce the degradation of the NFX-123 repressor and also stimulates Myc-induced hTERT transcriptional activation (Klingelhutz et al., 1996; Veldman et al., 2001; McMurray and McCance, 2003; Bedard et al., 2008). More recently, a study showed that E6 interacts directly with telomere DNA sequence and the hTERT protein (Liu et al., 2009). It was demonstrated that it also stimulates telomerase activity in a manner independent of its transcriptional activation of hTERT through these interactions. Hsp90 has been identified in a number of studies as a component of the telomerase complex and is necessary for its activity (Holt et al., 1999; Forsythe et al., 2001; Keppler et al., 2006; Toogun et al., 2008; Woo et al., 2009). However another finding has reported that Hsp90 binds directly to the hTERT promoter to actively stimulate hTERT transcription (Kim et al., 2008). Telomerase regulation appears to be a very stringently controlled process especially in humans. By exerting a dual mode of regulation (via telomerase assembly and hTERT transcriptional activation), RuvBl2 can switch on the telomerase activation

pathway more rapidly than would be the case if it just influenced one particular stage of the pathway.

The proteomics coverage in the project was not as highly extensive as originally intended; a contributing factor to this may have been the suppression of the signal of putative telomerase regulators by more abundant proteins. This may have obstructed detection of TFs previously known to control hTERT transcription. Targeted mass spectrometry allows for the selective identification of less abundant proteins based on their known proteotypic peptides (Picotti et al., 2008, 2009). Such an approach integrated with SILAC could have facilitated a more extensive quantitative comparison of the TFs known to be involved in telomerase regulation. Although this study provides evidence that RuvBl2 participates in hTERT transcriptional activation, further experimentation could potentially highlight in greater detail the underlying mechanisms involved. In particular, it would be interesting to ascertain through micrococcal nuclease mapping assays if the RuvBl2 interaction (due to its ATPase activity) can lead to chromatin remodelling. Given that RuvBl2 is not known to possess DNA-binding activity, it is also necessary to determine its precise mode of recruitment to the hTERT promoter. Although it is known to interact with c-Myc, interaction studies with this and other hTERT promoter TFs would clarify this issue. ChIP analyses targeting histone post-translational modifications such as acetylation should also be performed to discover if RuvBl2 participates in inducing these as a subunit of the TIP60 HAT complex.

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Appendices

Appendix 1. Relative quantitation of protein expression in 6G and 1-3C cells using SILAC to detect POM protein of interest.

Description	Accession no.	Mass[kDa]	6G/1-3C
ADP/ATP translocase1(Adenine nucleotide translocator 1) (ANT 1) (ADP,ATP carrier protein 1)	ADT1_HUMAN	33.28	0.9
Afadin (Protein AF-6) - Homo sapiens (Human)	AFAD_HUMAN	206.58	0.42
Antigen KI-67 - Homo sapiens (Human)	KI67_HUMAN	360.74	0.91
ATP-dependent RNA helicase A (EC 3.6.1.-)(DNA helicase II) (NDH II) (DEAH box protein 9)	DHX9_HUMAN	142.2	1.66
Chromodomain helicase-DNA-binding protein 4 (E 3.6.1.-) (ATP-dependent helicase CHD4) (CHD-4)	CHD4_HUMAN	219.42	1.26
Chromosome-associated kinesin KIF4A (Chromokinesin) - Homo sapiens (Human)	KIF4A_HUMAN	141.42	0.72
Clathrin heavy chain 1 (CLH-17) - Homo sapiens (Human)	CLH1_HUMAN	191.49	0.84
Coatomer subunit alpha (Alpha-coat protein) (Alpha-COP) (HEPCOP) (HEP-COP)	COPA_HUMAN	139.81	0.88
Cytoskeleton-associated protein 5 (Colonic and hepatic tumor over-expressed protein)	CKAP5_HUMAN	225.37	0.77
DNA (cytosine-5)-methyltransferase 1 (EC 2.1.1.37 (Dnmt1) (DNA methyltransferase Hsal)	DNMT1_HUMAN	185.43	0.81
DNA mismatch repair protein MSH6 (MutS-alpha 1 kDa subunit) (G/T mismatch-binding protein) (GTBP)	MSH6_HUMAN	154.55	0.92
DNA repair protein RAD50 (EC 3.6.-.-) (hRAD50) - Homo sapiens (Human)	RAD50_HUMAN	153.8	1.17
DNA topoisomerase 2-alpha (EC 5.99.1.3) (DNA topoisomerase II, alpha isozyme) - Homo sapie (Human	TOP2A_HUMAN	175.03	1.08
DNA topoisomerase 2-beta (EC 5.99.1.3) (DNA topoisomerase II, beta isozyme) - Homo sapiens (Human)	TOP2B_HUMAN	184.14	1.05
DNA-dependent protein kinase catalytic subunit C (DNA-PK catalytic subunit) (DNA-PKcs)	PRKDC_HUMAN	473.83	1.66
Dynactin-1 (150 kDa dynein-associated polypeptid (DP-150) (DAP-150) (p150-glued) (p135) - Homo sapiens	DYNA_HUMAN	141.61	0.66
Dynein heavy chain, cytosolic (DYHC) (Cytoplasmic dynein heavy chain 1) (DHC1) (Dynein heavy chain)	DYHC_HUMAN	534.86	0.85
E3 SUMO-protein ligase RanBP2 (Ran-binding protein 2) (Nuclear pore complex protein Nup358)	RBP2_HUMAN	362.44	1.53

Appendix 1 (Continued).

Description	Accession no.	Mass[kDa]	6G/1-3C
Eukaryotic translation initiation factor 3 subunit 10 (3 theta) (eIF3 p167) (eIF3 p180)	IF3A_HUMAN	166.87	1.1
Eukaryotic translation initiation factor 5B (eIF-5B) (Translation initiation factor IF-2) - Homo sapiens	IF2P_HUMAN	139.18	0.7
FACT complex subunit SPT16 (Facilitates chromatin transcription complex subunit SPT16) (hSPT16)	SPT16_HUMAN	119.84	1.71
Fatty acid synthase (EC 2.3.1.85) [Includes: [Acyl-carrier-protein] S-acetyltransferase (EC 2.3.1.3)	FAS_HUMAN	275.9	0.86
Filamin-A (Alpha-filamin) (Filamin-1) (Endothelial actin-binding protein) (Actin-binding protein 28)	FLNA_HUMAN	283.35	0.97
Filamin-B (FLN-B) (Beta-filamin) (Actin-binding-like protein) (Thyroid autoantigen)	FLNB_HUMAN	280.23	0.58
Filamin-C (Gamma-filamin) (Filamin-2) (Protein FLNc) (Actin-binding-like protein) (ABP-L)	FLNC_HUMAN	293.39	1.01
GCN1-like protein 1 (HsGCN1) - Homo sapiens	GCN1L_HUMAN	295	1.27
Host cell factor (HCF) (HCF-1) (C1 factor) (VP16 acces protein) (VCAF) (CFF)	HCFC1_HUMAN	210.74	1.26
LIM domain only protein 7 (LOMP) (F-box only protein Homo sapiens (Human)	LMO7_HUMAN	194.04	1.07
Mediator of DNA damage checkpoint protein 1 (Nuclea factor with BRCT domains 1) - Homo sapiens (Hum	MDC1_HUMAN	227.69	1.52
Microtubule-associated protein 1B (MAP 1B) [Contains MAP1 light chain LC1] - Homo sapiens (Human)	MAP1B_HUMAN	271.67	1.06
Microtubule-associated protein 4 (MAP 4) - Homo sapiens (Human)	MAP4_HUMAN	121.47	0.93
Myb-binding protein 1A - Homo sapiens (Human)	MBB1A_HUMAN	149.75	1.34
Myosin phosphatase Rho-interacting protein (Rho-interacting protein 3) (M-RIP) (RIP3) (p116Rip)	MRIP_HUMAN	116.37	0.58
Myosin-10 (Myosin heavy chain, nonmuscle IIb) (Nonmuscle myosin heavy chain IIb) (NMMHC II-b) (NMMH)	MYH10_HUMAN	229.84	0.45
Myosin-9 (Myosin heavy chain, nonmuscle IIa) (Nonmuscle myosin heavy chain IIa) (NMMHC II-a)	MYH9_HUMAN	227.67	0.82
Myosin-Ib (Myosin I alpha) (MMI-alpha) (MMIa) (MYH-1c) -Homo sapiens (Human)	MYO1B_HUMAN	131.9	0.31
Neuroblast differentiation-associated protein AHNAK (Desmoyokin) (Fragments) - Homo sapiens (Human)	AHNK_HUMAN	312.58	0.89
Nuclear mitotic apparatus protein 1 (NuMA protein) (SP-H antigen) - Homo sapiens (Human)	NUMA1_HUMAN	239.23	1.22
Nuclear mitotic apparatus protein 1 (NuMA protein) (SP-H antigen) - Homo sapiens (Human)	NUMA1_HUMAN	239.23	1.03
Nucleoprotein TPR - Homo sapiens (Human)	TPR_HUMAN	265.85	1.86

Appendix 1 (Continued).

Description	Accession no.	Mass[kDa]	6G/1-3C
Paired amphipathic helix protein Sin3a (Transcriptional corepressor Sin3a) (Histone deacetylase comlex)	SIN3A_HUMAN	145.09	0.94
Plectin-1 (PLTN) (PCN) (Hemidesmosomal protein 1) (HD1) -Homo sapiens (Human)	PLEC1_HUMAN	533.44	2.44
Pre-mRNA-processing-splicing factor 8 (Splicing factor Prp8) (PRP8 homolog)	PRP8_HUMAN	274.76	0.93
Probable global transcription activator SNF2L4 (ATP-dependent helicase SMARCA4)	SMCA4_HUMAN	185.11	0.65
Proline-, glutamic acid- and leucine-rich protein 1 (Modulator of nongenomic activity c estrogen receptor)	PELP1_HUMAN	120.9	1.64
Protein flightless-1 homolog - Homo sapiens (Human)	FLII_HUMAN	144.66	0.74
Protein phosphatase 1 regulatory subunit 12A (Myosin phosphatase-targeting subunit 1) (Myosin phosphatase	MYPT1_HUMAN	115.21	0.77
Ras GTPase-activating-like protein IQGAP1 (p195) - Homo sapiens (Human)	IQGA1_HUMAN	189.77	0.95
Replication factor C subunit 1 (Replication factor large subunit) (RF-C 140 kDa subunit)	RFC1_HUMAN	128.18	1.15
Spectrin alpha chain, brain (Spectrin, non-erythroid alpha chain) (Alpha-II spectrin)	SPTA2_HUMAN	285.18	0.85
Spectrin beta chain, brain 1 (Spectrin, non-erythroid beta chain 1) (Beta-II spectrin)	SPTB2_HUMAN	275.25	0.78
Splicing factor 3B subunit 1 (Spliceosome-associated protein 155) (SAP 155) (SF3b155) (Pre-mRNA-splicing factor)	SF3B1_HUMAN	145.72	0.95
Structural maintenance of chromosomes protein (SMC1alpha protein) (Sb1.8) - Homo sapiens (Human)	SMC1A_HUMAN	143.14	0.94
Structural maintenance of chromosomes protein (Chromosome-associated polypeptide C) (hCAP-C)	SMC4_HUMAN	147.79	0.99
SWI/SNF-related matrix-associated actin-depenc regulator of chromatin subfamily C member 1	SMRC1_HUMAN	122.68	0.84
Talin-1 - Homo sapiens (Human)	TLN1_HUMAN	271.8	0.9
Telomere-associated protein RIF1 (Rap1-interacting factor 1 homolog) - Homo sapiens (Human)	RIF1_HUMAN	276.5	0.9
THO complex subunit 2 (Tho2) - Homo sapiens (Human)	THOC2_HUMAN	171.16	1.51
Thyroid receptor-interacting protein 12 (TRIP12) Homo sapiens (Human)	TRIPC_HUMAN	222.27	1.14
Treacle protein (Treacher Collins syndrome protein) - Homo sapiens (Human)	TCOF_HUMAN	152.25	0.98

Appendix 1 (Continued).

Description	Accession no.	Mass[kDa]	6G/1-3C
Tumor suppressor p53-binding protein 1 (p53-binding protein 1) (p53BP1) (53BP1) - Homo sapiens	TP53B_HUMAN	215.53	1.33
U5 small nuclear ribonucleoprotein 200 kDa helicase (EC 3.6.1.-) (U5 snRNP-specific 200 kDa protein)	U520_HUMAN	246.04	0.92
Utrophin (Dystrophin-related protein 1) (DRP1) (DRP) – Homo sapiens (Human)	UTRO_HUMAN	396.51	0.63
Uveal autoantigen with coiled-coil domains and Ankyrin repeats protein - Homo sapiens (Human)	UACA_HUMAN	162.4	0.67
WD repeat protein 33 (WD repeat protein WDC146) – Homo sapiens (Human)	WDR33_HUMAN	146.24	1.21
Tumor suppressor p53-binding protein 1 (p53-binding protein 1) (p53BP1) (53BP1) - Homo sapiens	TP53B_HUMAN	215.53	1.33
U5 small nuclear ribonucleoprotein 200 kDa helicase (EC 3.6.1.-) (U5 snRNP-specific 200 kDa protein)	U520_HUMAN	246.04	0.92
Utrophin (Dystrophin-related protein 1) (DRP1) (DRP) – Homo sapiens (Human)	UTRO_HUMAN	396.51	0.63
Uveal autoantigen with coiled-coil domains and ankyrin repeats protein - Homo sapiens (Human)	UACA_HUMAN	162.4	0.67

Appendix 2. Peptides used to quantify proteins for the global nuclear SILAC comparison.

Protein	Peptide	Log(e)
Actin-like protein	QEYDESGPSIVHR	-3.2
General transcription factor II-I (GTFII-I)	FAQALGLTEAVK	-5.2
G-protein-signaling modulator 3	SEPPLPPGGQELLELLLR	-4.4
Heat shock-related 70 kDa Protein 2	STAGDTHLGGEDFDNR	-6.5
	VEIIANDQGNR	-4.4
	ARFEELNADLFR	-3.6
Heterogeneous nuclear ribonucleoprotein A1	LFIGGLSFETTDESLR	-10.4
	NQGGYGGSSS SSSYGSGR	-8.8
	IEVIEIMTDR	-3.2
heterogeneous nuclear ribonucleoprotein A1-like	LFIGGLSFETTDESLR	-10.4
Heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNP A2 / hnRNP B1)	LFIGGLSFETTEESLR	-2.9
Interferon-induced transmembrane protein 2	MVGDVTGAQA YASTAK	-6.3
IWS1 homolog (IWS1-like protein)	DGGTFISDADDVVSAMIVK	-7.2
L-lactate dehydrogenase B chain	IVADKDYSVT ANSK	-3.9
Myosin-11 (Myosin heavy chain 11)	QLLQANPILEAFGNAK	-6.8
Myosin-1a	EQLLQSNPVLEAFGNAK	-4.1
Peripherin	NLQEAEEWYK	-3.6
Ras-related protein Rab-1B	EFADSLGIPFLETSAK	-3.1
60S ribosomal protein L11	VLEQLTGQTP VFSK	-5
RuvB-like 2	TQGFLALFSGDTGEIK	-7.6
Signal transducer and activator of transcription 1-alpha/beta	TFSLFQQLIQSSFVVER	-3.8
Succinate dehydrogenase	LGANSLLDLV VFGR	-3.1
Thyroid hormone receptor-associated protein 3	SIFQHIQSAQSQR	-3.1
Tubulin alpha-1B chain	AVFVDLEPTVIDEVR	-3.7
Ubiquinol-cytochrome-c reductase complex core protein 2, mitochondrial precursor	TIAQGNLSNTDVQAAK	-6.5
Ubiquitin-conjugating enzyme E2 N	TNEAQAIETA R	-4.3

Appendix 3. Residue modification parameters used in GPM protein search.

6.0201@K,10.0083@R,0.98401@N,79.9663@S,79.9663@T,79.9663@Y,15.999@M,15.999
@W,0.98401@Q,71@C,14.016@D,14.016@E,14.016@H

14.016@N,31.9898@M,31.9898@W,
18.011@S,18.011@T,14@C,14.016@Q,14.016K,14.016@R

20.03612903@K,24.0242686@R